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TITLE: Angiogenesis Research to Improve Therapies for Vascular Leak Syndromes, Intra-abdominal Adhesions, and Arterial Injuries

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14. ABSTRACT

The three goals of this project are: (i) to discover and develop novel drugs which could prevent or reverse the vascular leak syndrome; (ii) to develop angiogenesis inhibitors which would inhibit post-operative abdominal adhesions; and, (iii) to isolate endothelial progenitor cells from blood, capable of being expanded in vitro and applied to vascular grafts. Progress has been made in each category: we have demonstrated suppression of vascular leak by Caplostatin in response to the pro-angiogenic agent VEGF; we have demonstrated that celebrex has an effect on adhesion prevention, and we have two other angiogenesis inhibitors that we would now like to test; and, we have identified cells from cord blood and adult bone marrow that can substitute for mature smooth muscle cells isolated from a healthy vein.

15. SUBJECT TERMS
endostatin; Caplostatin; angiogenesis; angiogenesis inhibitors; celecoxib; adhesions； circulating endothelial cells; endothelial progenitor cells; mesenchymal stem cells; tissue-engineering.

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Table of Contents

Project I: New Therapy for Vascular Leak Syndromes

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I. INTRODUCTION:

This past year we have been investigating the efficacy and mechanism by which two known angiogenesis inhibitors, Caplostatin and endostatin, inhibit vascular permeability of capillary endothelial cells. The goal of this research is to develop novel therapies to prevent and reverse vascular leak syndromes. These syndromes commonly affect soldiers on the battlefield and result from blast injuries, crush injuries, and smoke inhalation, among others. Complications from vascular leak syndromes include tissue edema or swelling after injury and often lead to the loss of the use of limbs or other affected organs.

We have been utilizing angiogenesis assays and mouse models to determine whether the angiogenesis inhibitors Caplostatin and endostatin prevent vascular leak artificially induced by three different agonists. Similarly the use of transgenic mouse models has allowed us to being exploring the mechanism by which these agents work.

II. BODY:

As outlined in the Statement of Work, our research accomplishments this past year include the following:

I. Produce sufficient TNP-470-HPMA, endostatin, and its peptide, for entire project.

- We have now succeeded in optimizing the large-scale production of TNP-470-HPMA, or Caplostatin. This past year we have spent a considerable amount of time troubleshooting the scale-up production of Caplostatin. Our initial attempts at large-scale production led to toxicity in our Caplostatin batches causing weight loss in mice treated with Caplostatin for 2 weeks. Throughout this past year, we systematically changed each variable to determine the cause of the toxicity. As detailed in our third quarter report, we have now optimized large-scale production of Caplostatin resulting in batches that do not cause toxicity.

- We have now confirmed the stability of an Fc-conjugated N-terminus endostatin peptide. As outlined in our third quarter report, the pharmacokinetics of Fc-endostatin demonstrates significant levels of circulating Fc-endostatin up to 12 days after

intraperitoneal injection. Production of purified Fc-endostatin is prepared by an outside vendor therefore there are no limits to the amounts we are able to obtain.

*II. Carry out Miles test using either TNP-470-HPMA, or endostatin, or its peptide to determine if these can **prevent** anti-permeability activity.*

• We have performed the Miles permeability assay with mice after daily Caplostatin (TNP-470-HPMA) treatment for 4 days. The agonists used in these assays include the pro-angiogenic protein VEGF, along with histamine, PAF and saline as a control. Our most recent studies (performed during the 4th quarter) have utilized our Caplostatin batches that lacked toxicity. These studies have preliminarily demonstrated that Caplostatin protects against vascular permeability in response to VEGF in SCID (or immunocompromised) mice. We are currently confirming this data in larger numbers of mice and repeating these experiments in wild-type mice.

*III. Determine the mechanism by which Caplostatin significantly **reduces** circulating thrombospondin and circulating endostatin while at the same time causes inhibition or regression of tumor growth.*

• In response to requests from the DOD, we have limited anti-tumor studies performed with these antiangiogenic therapies. Since this project is focusing on the prevention of vascular leak syndromes, we have utilized tumor regression as a means to assess the toxicity of Caplostatin or endostatin after long-term treatment.

• Studies to examine the mechanism of Caplostatin's function have been investigated by performing Miles permeability assays in thrombospondin1-null and endostatin (ie., Collagen XVIII)-null mice. Initial experiments suggest that the efficacy of Caplostatin may be reduced in the absence of endogenous thrombospondin-1 as illustrated by the lack significant protection against VEGF-induced vascular permeability. Preliminary experiments also indicate that endostatin may not be necessary for permeability protection by Caplostatin. Initial experiments in endostatin-null mice compared to wild-type mice demonstrate similar protection against vascular permeability in both groups.

III. KEY RESEARCH ACCOMPLISHMENTS:

• Optimized scale-up production of Caplostatin lacking toxicity.

• Demonstrated suppression of vascular leak by scaled-up batch of Caplostatin in response to the pro-angiogenic agent, VEGF.

• Initial studies suggest that the mechanism of Caplostatin's anti-permeability action may depend on the endogenous angiogenesis inhibitor thrombospondin-1 but not on endostatin.

• Demonstrated the stability of circulating Fc-conjugated endostatin peptide levels up to 12 days after initial injection.

IV. REPORTABLE OUTCOMES:

Manuscripts:

1. Folkman J. (2007). Angiogenesis: An Organizing Principle for Drug Discovery? **Nat Rev Drug Discov.** (in press).

Presentations:

1. Miami Nature Biotechnology Winter Symposium, Miami FL (Feb 4, 2006). "Endogenous Angiogenesis Inhibitors: Do The Molecules Suppress Angiogenesis-Dependent Disease?"

2. St. Jude Children's Research Hospital Seminar, Memphis TN (June 23, 2006). "Angiogenesis: An Organizing Principle in Medicine and Biology"

3. European VitreoRetina Society, Cannes France (Sept 7, 2006). "Potential future advances from research in tumor angiogenesis".

4. Mayo Clinic Cancer Center, Rochester MN (Oct 27, 2006). "Antiangiogenic Therapies: Past, Present, and Future".

5. Department of Defense, Fort Dietrick MD (Nov 16, 2006). "Angiogenesis research to improve vascular leak syndromes, intra-abdominal adhesions and arteriole injuries".

V. CONCLUSION:

Our studies optimizing the scale-up production of Caplostatin suggest that the production of this therapy will not be limiting for pre-clinical and initial clinical trials. Our work demonstrating the efficacy of Caplostatin in preventing vascular permeability further suggests that this anti-angiogenic agent may be used as a new therapy to prevent vascular leak syndromes suffered by soldiers such as chest or head injuries, crush injuries or inhalation of hot gasses or smoke. The use of the Miles permeability assay in mouse models with local vascular agonist will further allow us to optimize Caplostatin dosing schedules for the eventual transition to clinical application and use in the battlefield.

Our work demonstrating the stability of our Fc-conjugated endostatin peptide will allow us to examine the efficacy of this therapy in preventing local vascular permeability in our mouse models. These studies will determine whether Fc-endostain may be used in clinical application. Furthermore, the long-term stability of Fc-endostatin suggests that both delivery and use of this therapy may be significantly more feasible and cost-effective than any other currently utilized therapies.

"SO WHAT SECTION"

The significance of this work remains the same as in the initial grant application. The development of these novel non-toxic drugs, which may be administered to soldiers after receiving a number of types of injuries that trigger vascular leak syndromes, may protect and/or reverse complications sustained by these soldiers.

VI. REFERENCES:

None.

Project II: The Prevention of Post-Operative and Traumatic Abdominal and Non-abdominal Adhesions

Mark Puder, M.D. Ph.D., *Primary Investigator*
Sendia Kim, M.D., *Research Fellow*
Arin Greene, M.D., *Research Fellow*

I. INTRODUCTION

Several approaches have been attempted to inhibit intra-abdominal adhesion formation, most with limited success [3, 4]. While most studies into the prevention of adhesion formation have been focused on promoting fibrinolysis and inhibiting re-epithelialization of peritoneal surfaces, several investigators have implicated mediators of angiogenesis in the formation of adhesions. Because newly forming adhesions produce angiogenic factors that recruit new endothelium, COX-2 inhibitors are likely to target the expressed COX-2 enzyme on the newly proliferating vasculature. Vascular endothelial growth factor, fibroblast growth factor, and transforming growth factor beta are up-regulated during adhesion formation [5-7]. Because newly forming adhesions produce angiogenic factors that recruit new endothelium, COX-2 inhibitors are likely to target the expressed COX-2 enzyme on the newly proliferating vasculature. This effect was demonstrated by the significant decrease in microvessel density in the celecoxib treated animals from our murine model study [1]. In addition mediators of angiogenesis, such as VEGF, have been shown to be up-regulated during adhesion formation. Sunitinib is a tyrosine kinase inhibitor with anti-angiogenic properties acting primarily through the blockade of VEGF receptors 1 and 2 (VEGFR). Sorafenib has only been approved for clinical trials and inhibits VEGFR 2 and VEGFR 3. By blocking VEGFR, and thus counteracting the effects of VEGF upregulation, the formation of adhesions may be prevented.

Rabbits are more predictive of human disease and are the smallest large animals that have been used in testing adhesions. We have been using an adhesion model in rabbits to test the effects of celecoxib in adhesion prevention.

II. BODY

During the **first quarter** we began using a model in which the uterine horn of the rabbit was abraded and 2 sets of vessels in the mesometrium were tied off. A 5 cm lower midline incision was made in the abdomen. The abdomen was entered and the uterine horns were brought out through the incision. Using a 10 blade scalpel, the uterus and fallopian tubes were abraded until punctate hemorrhages appeared. Two small groups of collateral blood vessels were tied

off within the mesometrium using 5-0 silk ties. A pilot study performed in 2/06 demonstrated that this technique resulted in the formation of adhesions.

The table 1 demonstrates the results of the initial six rabbits.

3/24/06	Celebrex	Adhesions	total	Comments
1 (515)	Yes	Minimal, unilat, removed with blunt (3)	3	
2 (517)	Yes	Minimal, bil, removed w/blunt (3)	3	More than 515
3 (516)	Yes	Sig adhesions Fallopian tube-meso (3) Uterus-meso (3) FT-FT (3)	9	Did not eat Celebrex for the first 4 days
4 (519)	No	Small intestine-meso (4) Uterus-meso b/l (3) Ovary-meso uni (2)	9	
5 (518)	No	FT-FT unilat (4) FT-meso b/l (2) Uterus-meso uni (3)	9	
6 (514)	No	Uterus-meso b/l (2)	2	

The tenacity of the adhesions were scored as previously described (tenacity: 0=none, 1=fell apart, 2=lysed w/traction, 3=lysed w/blunt dissection, 4=lysed w/sharp dissection). The results from this study were encouraging. They demonstrated a positive effect of Celebrex in the prevention of adhesions for those rabbits that consumed the drug.

In the **second quarter** we used younger rabbits in hopes that they would be more easily trained to consume the drug. By allowing the rabbits to adjust to the housing facility for a longer period of time (7days) and training them to eat the applesauce for 7 days, we no longer had any problems with administration of the drug. We also used the youngest sexually mature rabbits available (5 months old) and found they were easier to train and feed.

Our preliminary results of our report on 4/06 were encouraging. Our repeat study on 5/06 revealed a trend towards adhesion reduction in the Celebrex group, but this was not statistically significant ($p=0.067$). Therefore we increased the dose of Celebrex from 75mg/kg/day to 100 mg/kg/day.

	Celebrex	% ut (ant+post)	tenacity	total
1 (544)	Yes	1	3	4
2 (546)	Yes	2	2	4
3 (549)	Yes	2	3	5
4 (545)	No	2	4	6
5 (547)	No	3	4	7
6 (548)	No	2	3	5

% ut covered
0=0, 1=25%, 2=50%, 3=100%

Tenacity
0=none, 1=fell apart, 2=lysed w/traction, 3=lysed w/blunt dissection, 4=lysed w/sharp dissection

In addition we performed tendon experiments to test if Celebrex was effective in preventing tendinous adhesions. In our initial experiment, rabbits were treated with Celebrex for 10 days at 75mg/kg/day (given twice a day) or a control of applesauce. The procedure was performed as follows: the rabbit was anesthetized with isoflurane. Its right hind leg was shaved from above the knee to the toes. The area was prepped with a betadine and alcohol wash. The leg was elevated and a tourniquet was placed. An incision was made from the metacarpal phalangeal joint to the proximal interphalangeal joint of the 2nd and 3rd toes. Tacking sutures were placed for exposure of the tendon. The tendon sheath was incised, carefully to avoiding the neurovascular bundle on each side. The pulleys above and below the flexor digitorum profundus (FDP) were kept intact. The FDP was divided and a 4 strand repair was performed. A Kessler repair was performed with a 5-0 nylon, then a mattress stitch was placed (5-0 nylon). The tendon sheath was closed with a 6-0 vicryl. Interrupted deep dermal stitches were placed with a 5-0 PDS, then a running 5-0 nylon was used to close the skin. Betadine was washed off the leg and gauze was placed between the toes. The leg was then wrapped with gauze and 2 tongue depressors were placed on the dorsum of the foot to prevent hyperextension. The leg was then immobilized with a cast covering the entire foot to above the knee with 3 inch plastor times 2. Rabbits #1-6 represent the 10 day experiment. The macroscopic adhesions were based upon a score by Healy, et al.[9]

Rabbit number		Macroscopic score	Left digit ROM	Right (repaired tendon) digit ROM
(1) 535 control	2nd	3	90	90
	3rd	3	90	90
(2) 536 control	2nd	3	90	90
	3rd	4	90	90
(4) 538 control	2nd	2	90	90
	3rd	2	90	90
(3) 537 Celebrex	2nd	3	90	90
	3rd	3	90	90
(5) 539 Celebrex	2nd	2	90	90
	3rd	2	90	90
(6) 540 Celebrex	2nd	3	90	90
	3rd	3	90	90
Repeat Celebrex treatment 21 days				
(7) 544 control	2nd	3	na	105
	3rd	3	na	110
(8) 546 control	2nd	3	Na	85
	3rd	3	Na	85
(9) 545 Celebrex	2nd	3	Na	120
	3rd	3	Na	105
(10) 547 Celebrex	2nd	3	Na	115
	3rd	3	Na	115

Adhesion and gliding scoring:
0=no adhesions w/ free tendon gliding
1=minimal adhesions, w/ tendon easily exposed by blunt dissection, minimal impairment of tendon gliding
2=mod adhesions, impairment of gliding
3=severe adhesions, tendon could be exposed by sharp dissection w/ grossly limited tendon gliding
4=maximum adhesions, inability to distinguish the tendon fr surrounding tissue on dissection and no gliding possible

Range of motion was assessed by measuring the angle of flexion with a smaller angle demonstrating improved range of motion (ROM). The non-operated tendon of the opposite foot was used as internal controls. No differences were noted between the Celebrex or control group. Therefore we decided to treat the animals for 21 days on Celebrex (75mg/kg/day) or control with leg immobilization. There were no differences between the Celebrex or control group, as seen in rabbits #7-10. The only improvement of function was seen in the control rabbit #8, who removed his cast on post operative day 10.

We decided to discontinue the tendon experiments, as there has been no difference demonstrated with the use of Celebrex for preventing tendinous adhesions.

In the **third quarter** we increased the dose of the drug to 100mg/kg/day (given twice a day), and had encouraging results (see below). The mean adhesion score for the control group was 6.333 and the mean adhesion score for the Celebrex group was 4.667 (p=0.024).

	Celebrex	% ut (ant+post)	tenacity	total
1 (553)	Yes	2	3	5
2 (554)	Yes	1	3	4
3 (555)	Yes	2	3	5
4 (556)	No	3	4	6
5 (557)	No	2	4	6
6 (558)	No	3	4	7

% ut covered
0=0, 1=25%, 2=50%, 3=100%

Tenacity
0=none, 1=fell apart, 2=lysed w/traction, 3=lysed w/blunt dissection, 4=lysed w/sharp dissection

There was a statistically significant improvement seen when the dose was increased, however it did not appear to be a clinically significant difference. In order to demonstrate an improved difference, we would like to treat our next group of rabbits at 150mg/kg/day. We have received approval by our animal care and use committee for this amendment, and are awaiting DOD approval.

In addition we have submitted a protocol to the DOD for the use of two other angiogenesis inhibitors, sunitinib and sorafenib. We have had encouraging results with the use of these angiogenesis inhibitors in adhesion prevention in a murine model, and would like to test them in a large animal model.

III. KEY RESEARCH ACCOMPLISHMENTS:

- Developed a model for adhesion formation in rabbits.
- Demonstrated that Celebrex does not have a significant effect in the prevention of tendinous adhesions in a rabbit model.

IV. REPORTABLE OUTCOMES:

None

V. CONCLUSION:

Several techniques have been attempted for developing a rabbit model of adhesions. Our initial experiment did not result in the formation of adhesions. The uterine horn model which we are currently using has resulted in consistent and reliable adhesion formation. We also tested Celebrex in the prevention of tendinous adhesions, and found it to have no effect in a rabbit model. In addition we have two other other angiogenesis inhibitors that demonstrated adhesion prevention a murine model, that we would like to test in a rabbit model.

"SO WHAT SECTION"

Our experiments suggest that Celebrex has an effect on adhesion prevention, and we are awaiting testing at a higher dosage to ensure effectiveness of the drug.

VI: REFERENCES:

1. Greene AK, Alwayn IP, Nose V, Flynn E, Sampson D, Zurakowski D, Folkman J ,Puder M (2005) Prevention of intra-abdominal adhesions using the antiangiogenic COX-2 inhibitor celecoxib. Ann Surg 242: 140-146
2. Kim S GA, Lee S, Arsenault D, Strijbosch R, Puder M (2006) Prevention of intra-abdominal adhesions with angiogenesis inhibition. Poster Session at Massachusetts Chapter of the American College of Surgeons Burlington, MA

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6. Chegini N (1997) The role of growth factors in peritoneal healing: transforming growth factor beta (TGF-beta). Eur J Surg Suppl: 17-23
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Project III: Creating New Blood Vessels using Blood-derived Endothelial and Mesenchymal Progenitor Cells

Joyce Bischoff, Ph.D., *Principal Investigator*
Juan Martin-Melero, Ph.D., *Research Fellow*

I: INTRODUCTION:

The hypothesis of this project is that autologous human endothelial progenitor cells (EPC) and mesenchymal cells from adult peripheral blood can be isolated, expanded, and used to create blood vessels and microvascular networks as needed for tissue engineered organs and tissues, and possibly for repair of damaged tissue. In previous work using sheep as a model system, we showed that endothelial progenitor cells (EPCs) isolated from a small amount of peripheral blood can be used to create non-thrombogenic long-lasting functional blood vessels (Kaushal et al., 2001). This encouraged us to develop methods for isolating EPC from human blood, and additionally mesenchymal stem cells (MSC) or smooth muscle progenitor cells (SMPCs) from blood, that we postulate can be used to form the smooth muscle layer in tissue engineered blood vessels. For preliminary studies, we have used umbilical cord blood to optimize methods for isolation and expansion as well as develop in vivo models for blood vessel formation. Methods are then adapted for peripheral blood from adults. This translation from cord blood to adult blood is a critical step because the numbers of EPCs and other types of progenitor cells are, as a rule, much lower in adult blood compared to cord blood. In summary, the overall goal in this project is to define the most straightforward and robust methods for isolating and expanding sufficient quantities of EPCs and SMPCs from adult peripheral blood, to test ability of these cells to form functional blood vessels in vivo and to optimize in vitro conditions so that rapid formation of functional vessels can be achieved.

II: BODY:

Technical Objective 1: To apply the methods we developed for isolating endothelial progenitor cells and mesenchymal stem cells from human cord blood to adult peripheral blood to determine feasibility of isolating autologous cells from a soldier's own blood

We have developed simple and robust methods for isolating human EPCs from cord blood and have adapted these to human adult peripheral blood. The adult EPCs show a similar phenotype to cord blood EPCs and adequate growth potential in vitro; 10^8 adult EPCs can be isolated from 50 ml of adult blood within 30 days. Importantly, we show that both cord blood EPCs and adult EPCs, when combined with mature human smooth muscle cells isolated from saphenous vein,

will form functional blood vessels in vivo within 7 days after implantation. A manuscript reporting these results in under revision for the journal Blood.

Our goal is to isolate a smooth muscle progenitor cell (SMPC) from blood that can substitute for the mature smooth muscle cells so that we can build blood vessels without sacrificing a healthy vessel. Using cord blood to start, we have been able to isolate a smooth muscle progenitor cell (SMPC) using methods similar to what we use for EPCs. The important step was to remove two endothelial growth factors, vascular endothelial growth factor-A (VEGF) and basic fibroblast growth factor (bFGF), as well as heparin, from the culture medium. The SMPCs (described in the 2006 2nd quarter progress report) grow rapidly in culture, express two smooth muscle markers α-SMA and calponin, and can be induced to express additional smooth muscle features by incubating with transforming growth factor-β. Experiments to isolate SMTPs from adult blood are underway. We are also investigating whether adult human bone marrow may contain SMPCs that will function in place of mature smooth muscle cells. (Human bone marrow and bone marrow MSC are purchased from Cambrex Bioproducts, Inc.) Bone marrow may provide an important, additional source of SMPCs.

Technical Object 2: <u>To determine if mesenchymal stem cells co-seeded with endothelial progenitor cells will induce microvessel formation in biodegradable scaffolds.</u>

These in vitro experiments have not yet been started because we moved to Technical Object 3, with some modifications, in order to gain evidence that the cells we are isolating do indeed have the ability to form blood vessels in vivo. Experiments in this objective will be carried out using a polyhydroxyalkanoate (PHA) non-woven scaffold that we have shown is suitable for adhesion and growth of endothelial cells (unpublished data) as well as poly-glycolic acid (PGA): poly-L-lactic acid (PLLA), a polymer we used in previous studies (Wu et al., 2004).

Technical Object 3: <u>To implant tissue-engineered microvascular networks into immune-compromised mice and determine if the tissue-engineered microvessels will form functional anastomoses with host vessels.</u>

Instead of implanting cells seeded on biodegradable scaffolds, which requires a surgical procedure, we developed a simple in vivo assay for testing ability of cells to form blood vessels in vivo so that we could more rapidly assess different sources of cells, effects of cell expansion, optimal ratios of EPCs to SMC, and effects of in vitro co-culturing. In this assay, cells to be tested are suspended in an extracellular matrix compound called Matrigel (commercially available) at 4°C. The cells/Matrigel suspension is injectrd subcutaneously into immune-deficient mice (nude mice). Matrigel solidifies almost instantly at 37°C, forming an implant containing the cells of interest. Our data show that within 7

days, the human EPCs and mature smooth muscle cells form blood vessels with functional connections to the mouse vasculature. In essence, we have in vivo vasculogenesis – the formation of blood vessels from cells that self-assemble. This is contrast to angiogenesis, which is the formation of new vessels from pre-existing vessels. However, we know that the human cells form blood vessels that make connections to the mouse vasculature because we can see red blood cells circulating through the human blood vessels

Our most recent data shows cord blood SMPCs can substitute for mature smooth muscle cells in this assay. Because this result establishes proof-of-principle that microvascular networks can be created in vivo with blood-derived progenitor cells, we have filed a provisional patent application with the U.S. patent office.

III: KEY RESEARCH ACCOMPLISHMENTS:

- Demonstrated cord blood EPCs combined with human saphenous vein smooth muscle cells form blood vessels in vivo within 7 days.
- Demonstrated adult peripheral blood EPCs combined with human saphenous vein smooth muscle cells form blood vessels in vivo within 7 days.
- Identified and optimized culture conditions for smooth muscle progenitor cells (SMPCs) from cord blood.
- Demonstrated cord blood SMPCs and cord blood EPCs form blood vessels in vivo within 7 days.
- Demonstrated that bone marrow-derived mesenchymal stem cells (commericially available) combined with cord blood EPCs form blood vessels in vivo within 7 days.

IV: REPORTABLE OUTCOMES:

Manuscripts:

Melero-Martin JM, Khan ZA, Picard A, Wu X, Paruchuri S and Bischoff J. 2007; In vivo vasculogenic potential of human blood-derived endothelial progenitor cells, i*n revision for Blood*

Abstracts:

Characterization of blood derived endothelial progenitor cells for tissue engineering applications, Juan Melero-Martin, Zia Khan, Sailaja Paruchuri, Xiao Wu and Joyce Bischoff;10th Biennial Meeting of the International Society for Applied Cardiovascular Biology March 8-11, 2006 La Jolla, CA

Presentations:

Joyce Bischoff, Seminar, CIMIT Forum, Massachusetts General Hospital, Boston, MA February 2006 Title: Endothelial Progenitors for Creating Blood Vessels

Joyce Bischoff, Seminar, Cardiovascular Research Center, Massachusetts General Hospital, Boston MA May 2006 Title: Endothelial Progenitor Cells for Cardiovascular Tissue-Engineering

Juan Melero-Martin, Speaker, International Society on Thrombosis and Haemostasis (ISTH): 52nd Scientific and Standardization Committee Annual Meeting, Oslo, Norway. June 2006 Title: Isolation, expansion and phenotypic characterization of EPCs for cardiovascular tissue-engineering.

Joyce Bischoff, Seminar, Angiogenesis Workgroup Meetings, University Hospital Maastricht, The Netherlands November 2006 Title: Endothelial Progenitor Cells for Cardiovascular Tissue-Engineering

Juan Melero-Martin, Seminar, Centro Andaluz de Biologia Molecular y Medicina Regenerativa (CAMIMER) Seville, Spain, December 2006, Title: Blood-derived progenitor cells for tissue vascularization.

Joyce Bischoff, Seminar, Oklahoma Medical Research Foundation, Oklahoma City, OK, Jan 18, 2007 Title: Endothelial Progenitor Cells for Cardiovascular Tissue-Engineering

Patents:

Title: Isolation and Expansion of Human Endothelial Progenitors from Cord Blood and Adult Peripheral Blood.
Inventors: Juan Melero-Martin and Joyce Bischoff
NP Ref 701039-058090-P
CMCC #1495
Filed: December 19, 2006

V: CONCLUSIONS:

Vascularization of engineered tissues is one of the major challenges of tissue-engineering (TE). We hypothesize that blood-derived endothelial progenitor cells (EPCs) have the required proliferative and vasculogenic activity to create vascular networks *in vivo*. To test this, EPCs isolated from human umbilical cord blood or from adult peripheral blood, and human saphenous vein smooth muscle cells (HSVSMCs) as a source of perivascular cells, were combined in Matrigel and implanted subcutaneously into immunodeficient mice. Evaluation of the implants at one week revealed an extensive network of human

specific lumenal structures containing erythrocytes, indicating the formation of functional anastomoses with the host vasculature. Quantitative analyses showed that the microvessel density was significantly superior to that generated by mature human dermal microvascular endothelial cells (HDMECs). We also found that as EPCs were expanded in culture, their morphology, growth kinetics and proliferative responses toward angiogenic factors progressively resembled those of HDMECs, indicating a process of *in vitro* cell maturation. This maturation correlated with a decrease of the degree of vascularization *in vivo*, which could be compensated by increasing the number of EPCs seeded into the implants. Our findings strongly support the use of human EPCs to form vascular networks in engineered organs and tissues. (Abstract from our submitted manuscript)

In addition to the studies in our submitted manuscript, we have shown that SMPC expanded from cord blood can substitute for HSVSMC. The next step will be to determine if we can isolate SMPCs from adult blood. We have also shown that bone marrow MSC (purchased from Cambrex) can substitute for HSVSM, suggesting bone marrow may be an alternative source of smooth muscle progenitor cells.

"SO WHAT SECTION"

Our results show that it is feasible to isolate EPCs from adult blood and form blood vessels in vivo. In addition, we have identified cells from cord blood and adult bone marrow that can substitute for mature smooth muscle cells isolated from a healthy vein. Therefore we have achieved a proof-of-principle – blood vessels can be created from blood-derived progenitor cells. The blood vessels are formed in Matrigel and form connections with the host vasculature. In the future, we would like to show that we can form vessels in vivo that will support tissue or organ function or facilitate tissue repair.

VI: REFERENCES:

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VII: APPENDICES:

Project I:

Folkman J. 2007. Angiogenesis: An Organizing Principle for Drug Discovery? **Nat Rev Drug Discov.** *(in press).*

Project II:

None

Project III:

Melero-Martin JM, Khan ZA, Picard A, Wu X, Paruchuri S and Bischoff J. 2007;
In vivo vasculogenic potential of human blood-derived endothelial progenitor cells, i*n revision for Blood*

Angiogenesis: an organizing principle for drug discovery?

Judah Folkman

Abstract | Angiogenesis — the process of new blood vessel growth — has an essential role in development, reproduction and repair. However, pathological angiogenesis occurs not only in tumour formation, but also in a range of non-neoplastic diseases that may possibly be classed together as "angiogenesis-dependent diseases". By viewing the process of angiogenesis as an 'organizing principle' in biology, intriguing insights into the molecular mechanisms of seemingly unrelated phenomena may be gained. This has important consequences for the clinical use of angiogenesis inhibitors and for drug discovery, not only for optimizing the treatment of cancer, but possibly also for developing therapeutic approaches for a variety of diseases that are otherwise unrelated to each other.

The term angiogenesis is generally applied to growth of microvessel sprouts the size of capillary blood vessels, a process orchestrated by a range of angiogenic factors and inhibitors (FIG.1). Although proliferating endothelial cells undergoing DNA synthesis are a common hallmark of angiogenic microvascular sprouts, extensive sprouts can grow for periods of time, mainly by migration of endothelial cells[1]. Physiologic angiogenesis is distinct from arteriogenesis and lymphangiogenesis and occurs in reproduction, development, and repair. It is usually focal, like blood coagulation in a wound, and self-limited in time, i.e., days (ovulation), weeks (wound healing), or months (placentation). In contrast, pathological angiogenesis may persist for years. Pathological angiogenesis is necessary for tumours and their metastases to grow beyond a microscopic size and it can give rise to bleeding, vascular leakage, and tissue destruction. These sequela of pathological angiogenesis can be responsible, directly or indirectly, for the symptoms, incapacitation, or death associated with a broad spectrum of "angiogenesis-dependent diseases"[2]. Examples of such diseases range from cancer to autoimmune diseases, age-related macular degeneration and artherosclerosis (TABLE 1).

The concept of "angiogenesis-dependent diseases" originated in 1972 with the recognition that certain non-neoplastic diseases, such as the chronic inflammatory disease psoriasis, depend on chronic neovascularization to provide a conduit for the continual delivery of inflammatory cells to the inflammatory site[3–5]. Subsequently, other non-neoplastic diseases were recognized to be in part angiogenesis-dependent, for example, infantile hemangiomas[6], peptic ulcers[7], ocular neovascularization[8], rheumatoid arthritis[9], and atherosclerosis[3,10,11]. This led to a more general understanding that the process of angiogenesis itself could be considered as an "organizing principle". While organizing principles are common in the physical sciences, they are now starting to be recognized in biology – other examples may be inflammation, or apoptosis, which are also aspects of many different otherwise unrelated diseases. The heuristic value of such a principle is that it permits connections between seemingly unrelated phenomena. For example, discovery of a molecular mechanism for one phenomenon may be more rapidly demonstrated for a second phenomenon, if one understands *a priori* that the two are connected. Furthermore, when the mechanisms underlying different diseases can be related in this way, the development of therapeutics for one disease could aid the development of therapeutics for others. Although it remains to be determined to what

extent treating pathologic angiogenesis in different “angiogenesis-dependent diseases” will be successful, the recent approval of ranibizumab (Lucentis) — an antibody fragment based on the anti-angiogenic cancer drug bevacizumab (Avastin) — for age-related macular degeneration suggests that such strategies merit investigation.

Here, I provide an overview of the current state of drug development of angiogenesis inhibitors, as well as certain drugs that have varying degrees of anti-angiogenic activity in addition to their other functions and highlight examples of anti-angiogenic strategies in unrelated diseases. Furthermore, I discuss burgeoning new directions in angiogenic research, the optimization of anti-angiogenic strategies, and how viewing angiogenesis as a possible organizing principle may uncover fruitful connections for future drug discovery.

A brief history of angiogenesis inhibitors

The attempt to discover angiogenesis inhibitors became possible after we and others had developed bioassays for angiogenesis during the 1970s. These included the long-term culture of vascular endothelial cells[12], the development of the chick embryo chorioallantoic membrane bioassay[13], the development of sustained release polymers[14], and the implantation of these polymers as pellets in the rabbit cornea[15] and murine cornea[16] to quantify the angiogenic activity of tumour-derived proteins.

The first angiogenesis inhibitors were reported in the 1980s from the Folkman laboratory, during a study that continued over twenty-five years[17,18] (Timeline). No angiogenesis inhibitors existed before 1980, and few scientists believed at that time that such molecules would ever be found. However, the effort to isolate and purify them was driven by preliminary data that led to the 1971 hypothesis that tumour growth is angiogenesis-dependent[19]. This effort was also informed by preliminary data that the removal of an angiogenic sustained release pellet from the rabbit cornea led to relatively rapid regression (weeks) of neovascularizaton induced by the pellet[14,20].

After the mid-1980s, we and others began to discover additional angiogenesis inhibitors[21–29] (Timeline). By the mid-1990s, new drugs with antiangiogenic activity entered clinical trials. These drugs began to receive FDA approval in the U.S. by 2003. Bevacizumab, which received FDA approval for colorectal cancer in 2004, was the first drug developed solely as an angiogenesis inhibitor[30]. However, certain nonendothelial cells (hematopoietic–derived cells which colonize tumour stroma and some cancer cells, such as in pancreatic cancer) can also express receptors for VEGF, raising the possibility that this drug may also have direct anti-tumour effects[129,135]. [AU:ok?] [AU:OK-YES] At this writing, 10 new drugs with antiangiogenic activity that is considered to be central to their therapeutic effects have been approved by the FDA in the U.S., and by equivalent agencies in 30 other countries, for the treatment of cancer and age-related macular degeneration (TABLE 2). At least 23 other drugs that have varying degrees of antiangiogenic activity are currently in clinical trials in the U.S. for different types of cancer, 7 of them in Phase III (TABLE 3). Other FDA approved drugs revealed antiangiogenic activity in addition to anti-cancer activity directed against tumour cells. For example, bortezomib (Velcade), approved as a proteasome inhibitor for the treatment of multiple myeloma, was subsequently demonstrated to also have potent antiangiogenic activity[31]

As the treatment range of angiogenesis inhibitors not only covers many different types of cancer, but also unrelated diseases such as macular degeneration and possibly others in the future, angiogenesis inhibitors or drugs that have varying degrees of anti-angiogenic activity may be defined as a class of drugs specifically targeting an ‘organizing principle’ in biomedicine.

Angiogenesis as a possible organizing principle: clinical applications

There are important clinical advantages to understanding angiogenesis as a possible organizing principle. For example, if a clinician recognizes that a patient’s disease may

be angiogenesis-dependent in part, it is conceivable that an angiogenesis inhibitor approved for one tumour type may be employed for a different tumour type, or even used off-label for a different disease.

An example of the former is the use of bevacizumab in colorectal cancer and also in non-small cell lung cancer, and an example of the latter is its off-label use for age-related macular degeneration. [AU:ok?] [AU: OK] Oncologists may also benefit from knowing that certain anti-cancer drugs originally developed to target cancer cells also have antiangiogenic activity. An example is cyclophosphamide.

A connection between colorectal cancer and macular degeneration

Bevacizumab is an antibody that neutralizes VEGF (vascular endothelial growth factor), and was approved by the FDA for colorectal cancer in 2004 (REF. 32). Ranibizumab (Lucentis) is a fragment of bevacizumab. In randomized clinical trials, ranibizumab injected into the eye at monthly intervals showed dramatic success in patients with age-related macular degeneration. In patients who were legally blind with an average visual acuity of ~20/300, approximately 40% recovered their sight and improved to a visual acuity of 20/40 (sufficient for some to drive a car). In ~90–95% of all patients, the disease was arrested, and there was no further loss of sight. In contrast, sham treated patients continually lost visual acuity over a 12-month period, as was expected[33–37, 38] (FIG. 3a). Macugen (pegaptanib) an anti-VEGF aptamer, was the first anti-VEGF drug to be approved by the FDA (2004) for the treatment of age-related macular degeneration. Over 75,000 patients with age-related macular degeneration have been treated with Macugen since its approval, and in the last year over 50,000 patients have been treated with either intravitreal Lucentis or off-label Avastin.

This may be the first time that a relatively non-toxic, anti-cancer drug has been injected into the eye to treat ocular neovascularization. It is rare to treat diseases as divergent as colorectal cancer and age-related macular degeneration with the same agent – except that the target for each was known to be VEGF[39–42].

Discovery of dual roles for cancer drugs

The cancer drugs erlotinib (Tarceva), cetuximab (Erbitux), and ZD6474 were originally developed as inhibitors of the epidermal growth factor receptor tyrosine kinase. For this reason, they are also known as anti-oncoprotein signal transduction inhibitors[43]. However, they were subsequently found to also inhibit tumour angiogenesis by blocking the VEGF receptor. Cetuximab, an anti-EGFR agent, produces an anti-tumour effect in vivo that is due both to the direct blockade of the EGFR-dependent mitogenic pathway and in part to the inhibition of secretion of various pro-angiogenic proteins such as VEGF, bFGF, TGF-alpha[130]

[43]. With this knowledge of their dual role[43] these drugs may be employed more effectively by oncologists who could follow guidelines for dose-efficacy of angiogenesis inhibitors, which differ from conventional cytotoxic chemotherapy (see below).

Angiogenesis as an organizing principle: emerging research directions

The usefulness of recognizing an underlying organizing principle during angiogenesis research is illustrated by several fascinating insights into diverse biological processes. Some examples of this are novel insights into platelet biology[44], metastasis[22], endothelial control of tissue mass[65,66], the concept of oncogene-dependence[80] and the surprising finding that some of the ligand-receptor pairs that mediate axon pathway finding also mediate angiogenesis[47]. In addition, genetic variations in expression of angiogenic proteins between different groups of individuals[86] provide further clues about the role of these angiogenesis regulatory proteins in different diseases.

Endothelium and neurons share regulatory proteins

In 1998, Michael Klagsbrun reported that neuropilin, a cell surface protein originally identified as a receptor for a signal that guides growing nerves, is also a receptor for

VEGF[45,46]. This marked the beginning of a merger between the fields of neural guidance and angiogenesis. It was discovered that a variety of ligand-receptor pairs that mediate axon pathway finding also mediate angiogenesis[47] (TABLE 4).

Also, during development, sensory nerves determine the pattern of arterial differentiation in blood vessel branching in the skin[48]. It was found that in the highly vascular dorsal root ganglia, neuronal VEGF interacts with endothelial cell VEGF receptor-2 (REF. 49). As the interactions of growth and motility proteins for neurons and endothelial cells are gradually uncovered, they may play important roles in future drug discovery, for example for drugs that can repair spinal cord injuries, or reverse Alzheimer's disease, or broaden the efficacy of currently approved angiogenesis inhibitors.

New platelet biology

In a 2001 review, we assembled the reports which showed that most of the endogenous angiogenesis regulatory proteins known at that time were contained in platelets or were on the platelet surface[50] (TABLE 5). Klement et al. subsequently reported that circulating platelets in mice take up and sequester angiogenesis regulatory proteins, such as VEGF, bFGF, platelet factor 4, and connective tissue-activating peptide (CTAP), when a microscopic sized human tumour is present in a mouse[51–53].

The angiogenic regulatory proteins are sequestered in alpha granules of platelets at a significantly higher concentration than in plasma. In fact, when radio-labeled VEGF is implanted subcutaneously in a Matrigel pellet in mice, platelet lysates take up virtually all of the radiolabeled VEGF and none is found in plasma[51]. Mouse platelets live ~3 - 4 days. Nevertheless, platelets appear to recycle the angiogenesis regulatory proteins they have scavenged, because the concentration of these proteins increases in the platelets over time (weeks to months), as long as the source of an angiogenesis regulatory protein is present. Also, a single intravenous injection of thrombospondin-1 (2μg) into thrombospondin-1 null mice continues to appear in platelet lysates for weeks [Sandra Ryeom, personal communication]. Furthermore, it was recently reported that in cancer patients receiving bevacizumab, the antibody was taken up by platelets where it was bound to VEGF[54].

This new platelet property, quantifiable by mass spectroscopy of platelet lysates, may permit the development of a biomarker for very early detection of tumour recurrence. In tumour-bearing mice, the platelet angiogenesis proteome detects microscopic tumours at a millimeter size, before they have become angiogenic, but when they are elaborating angiogenic proteins (VEGF, bFGF and PDGF), and antiangiogenic proteins (platelet factor 4, endostatin, or thrombospondin-1)[51,55].

Joseph Italiano has recently discovered that angiogenesis regulatory proteins are in fact segregated among two sets of alpha granules in platelets**:** positive regulators of angiogenesis in one set of alpha granules and negative regulators in the other set[56]. This heretofore unknown function of platelets links them with the process of angiogenesis. A new opportunity lies ahead to determine if and how platelets release pro-angiogenic proteins at a wound site and then later release antiangiogenic proteins. Furthermore, the putative role of platelet release of angiogenesis-regulatory molecules in tumours now remains to be elucidated. It may also be possible to develop drugs that selectively release antiangiogenic proteins from platelets trapped in hemangiomas or in cancer. It is likely that in the future, novel angiogenesis regulatory molecules that could be developed into drugs will be discovered in platelets.

A new mechanism for site specificity of metastasis

It is known that thrombospondin-1 is a potent angiogenesis inhibitor[57], expressed by fibroblasts and other stromal cells in many tissues. It is also clear that reduction of thrombospondin-1 expression in a tumour bed is a necessary pre-requisite for induction of neovascularization and for a microscopic tumour to become neovascularized and grow[58,59]. Randolph Watnick recently found that certain human tumours produce a

novel protein that specifically represses thrombospondin-1 in the stromal tissue to which the tumour is subsequently able to metastasize[60]. Suppression of antiangiogenic activity at the future metastatic site facilitates the initiation of angiogenesis by metastatic tumour cells. If this discovery can be generalized to other tumours, it could be the basis for the development of drugs, such as antibodies that could neutralize the thrombospondin-1 suppressor protein elaborated by the primary tumour.

When a new angiogenesis-based metastatic mechanism is uncovered, it is prudent to ask whether the new cancer mechanism could have a physiological counterpart. Similar to other normal tissues, thrombospondin-1 is highly expressed under normal conditions in the endometrium[61]**.** It is not known if thrombospondin-1 is suppressed prior to implantation of a fertilized ovum or of a blastocyst, and if so, by what mechanism, which is a topic of current investigation. There are potential clinical implications. More than 10% of all pregnancies miscarry early in the first trimester. Some women have repeated early miscarriages and are unable to carry a baby to term. In vitro fertilization often requires multiple cycles of ovum implantation. Could these problems be the result of insufficient suppression of endometrial thrombospondin-1, or of some other endogenous angiogenesis inhibitor in the endometrium? If so, could this condition be diagnosed by measuring thrombospondin-1 in the vaginal fluid? Could endometrial thrombospondin-1 then be suppressed, for example by a vaginal suppository containing a putative short-acting thrombspondin-1 suppressor protein? Another intriguing finding is that hemangiomas, benign tumours of infancy, display the gene signature of cells of the fetal placental endothelium, implying that they may ~~be metastasis from the placenta~~ originate from the fetal placenta.[62,63] (BOX 1). As hemangiomas usually regress spontaneously, they may reveal important clues about the molecular mechanisms of spontaneous regression of new blood vessels.

Endothelial cell control of tissue mass

When approximately 70% of the liver is removed in a rat (hepatectomy), the original mass regenerates completely in approximately 10 days[64]. Hepatocyte proliferation and endothelial cell proliferation are initiated the day after surgery. At approximately the 8th day, there is a wave of endothelial cell apoptosis, following which hepatocyte proliferation ceases[65]. The liver stops growing at ~10 days. However, if an angiogenic protein, such as VEGF or bFGF, is administered systemically, endothelial cells continue to proliferate and the liver continues to grow beyond its normal size. In contrast, if a specific inhibitor of endothelial proliferation is administered, liver regeneration is prevented, and the liver remains at 30% of its normal size. Discontinuation of the endothelial inhibitor is followed immediately by liver regeneration that is complete by 10 days[65]. These experiments indicate that normal tissue and organ regeneration are controlled in part by microvascular endothelium.

Growth and regression of fat is also controlled by endothelial proliferation or apoptosis, respectively[66]. Leptin-deficient mice gain up to approximately 1 gram per day, which is mainly fat. Adipocyte enlargement and proliferation is accompanied by endothelial proliferation restricted to fat. Systemic administration of an angiogenesis inhibitor (TNP-470 or endostatin), specifically induces endothelial apoptosis and a decrease in fat accompanied by rapid weight loss. When normal weight for age is reached, weight loss stops. A similar result is obtained when endothelial cells in fat are specifically targeted by a genetically regulated inhibitor of proliferation[67]. Growth of normal prostate is also under endothelial control[68], and so is bone growth[69]. Therefore, it appears that microvascular endothelial cells may control tissue mass, regardless of whether the cells in this mass have a normal genome or a cancer genome[70]. This raises a provocative question: “Is there some type of set-point or feed-back mechanism in endothelial cells that tells them when a normal organ such as liver, has reached its normal mass? If so, do tumour cells over-ride this mechanism, and how?”

What are the implications of this general principle for drug discovery? There is the possibility that specific endothelial inhibitors may be used to control obesity[67], as well as overgrowth of other tissues, i.e., uterine fibroids, overgrowth of bone caused by lymphangiogenesis, ectopic bone growth (fibrodysplasia ossificans progressiva)[71], and vascular malformations which grow rapidly after puberty, or after attempts at surgical excision, and for which there are currently no drugs. The endocrine-specific angiogenesis regulatory proteins, such as BV8 (REF. 72) (testicular cancer-specific), are of particular interest.

Is oncogene dependence angiogenesis-dependent?

The recognition that endothelial cells control tumour mass is critical for a more complete understanding of how oncogenes initiate tumour growth. The conventional wisdom is that oncogene activation in a cell leads directly to the formation of large, lethal tumours in mice. This concept is reinforced by experiments in which Ras or Myc oncogenes, under the control of the doxycycline promoter, induce rapid tumour growth when the oncogene is activated and lead to rapid tumour regression when the oncogene is inactivated[73–78]. This phenomenon is called 'oncogene-dependence' or 'oncogene-addiction'[79]. However, we have found that during oncogene induced tumour growth there is intense tumour angiogenesis associated with suppression of thrombospondin-1 in the tumour bed. When an oncogene is inactivated, the expression of thrombospondin-1, a potent angiogenesis inhibitor, is markedly increased in the tumour bed, leading us to propose that oncogene-addiction is angiogenesis-dependent[78]. This hypothesis has now been proved by deletion of thrombospondin-1 in the tumour and the host. In these mouse models, an activated oncogene induces more rapid tumour growth than in wild-type mice, but tumours do not regress after inactivation of the oncogene[77]. Restoration of thrombospondin-1 expression in the tumour results in tumour regression upon oncogene inactivation[80].

How could this change in thinking about oncogene-addiction provide new opportunities in drug discovery? Conventional wisdom (FIG. 4) implies that development of drugs targeted against oncogenes should be sufficient to control cancer. In fact, imatinib, which targets the product of the *bcr-abl* oncogene has demonstrated proof of concept by its success in the treatment of chronic myeloid leukaemia**.** In addition, imatinib targets the product of the oncogene c-Kit, and has also proved successful in treating gastrointestinal stromal tumour (GIST) in which this protein has a key role[81,82]. However, many patients eventually develop drug resistance[132], and there are numerous other oncogenes that could be responsible for inducing expression of redundant growth factors in these tumours. The imatinib experience also suggests that drugs will need to be developed against combinations of many oncogenes. A single angiogenesis inhibitor, especially a broad spectrum angiogenesis inhibitor, such as endostatin[28] or Caplostatin, or a combination of angiogenesis inhibitors, might block the effect of a large family of oncogenes, as the blockade of angiogenesis can prevent tumour growth downstream of oncogene activation. In fact, analysis of 15 of the most well studied oncogenes, revealed that the majority of them increase expression of VEGF (and or bFGF), and decrease expression of thombospondin-1 in tumour cells[83,84].

Genetic regulation of angiogenesis

Although we all carry endogenous angiogenesis inhibitors in our blood and tissues (at least 29 at this writing)[24,25,85], different individuals reveal distinct genetic differences in their angiogenic response to a given stimulus. For example, individuals with Down syndrome are protected against diabetic retinopathy, although they have a similar incidence of diabetes as individuals without Down syndrome[86,87]. They also have higher levels of circulating endostatin (~1.6 fold) than normal individuals because of an extra copy of the gene for the endostatin precursor (collagen XVIII) on chromosome 21 (REFS 87,88). Interestingly, they appear to be among the most protected of all humans against cancer. While testicular cancer and a megakaryocytic leukaemia have been reported for

individuals with Down Syndrome, they have the lowest incidence of the other ~200 human cancers compared to age-matched controls[86,87]. Conversely, individuals with a polymorphism in endostatin, arginine substituted for alanine at N104, have a significantly higher risk of breast cancer[89]. The correlation between endostatin levels and cancer susceptibility was demonstrated in mice - mice engineered to genetically overexpress endostatin to mimic individuals with Down syndrome have tumours that grow 300% slower[90], and in mice deleted of thrombospondin-1, tumours grow approximately 300% more rapidly, and faster still if two angiogenesis inhibitors are knocked out (tumstatin and thrombospondin-1)[90].

Another interesting finding is that African Americans rarely develop the wet form of age-related macular degeneration. They usually do not have intravitreal hemorrhages, and do not go blind from the 'dry' form of this disease[93]. In contrast, African Americans have a similar incidence of diabetic retinopathy. In macular degeneration neovascularization is in the choroidal layer that is surrounded by melanocytes containing melanin. In diabetic retinopathy neovascularization arises from the retina. The retinal pigmented epithelial cells contain a lighter form of oxidized melanin which differs from melanin in the choroid or in the skin. Also, African American infants rarely develop cutaneous hemangiomas in contrast to white infants.

These correlations suggest that factors linked to pigmentation/melanin are producing an inhibitory influence on the angiogenic balance in the melanin-rich tissues. However, there is no melanin in prostate or breast, and African Americans are not protected from cancer of these organs.

This hypothesis was examined in animal experiments. When the gene for tyrosinase (in the melanin pathway) was deleted from mice, the albino relatives C57Bl/6J-Tyr$^{c\text{-}2J}$ developed intense iris neovascularization and hemorrhage (hyphema) compared to weak neovascularization and no hemorrhage in the pigmented iris of wild-type mice. As expected, the amount of corneal neovascularzation was not significantly different between these two strains, because the cornea is not a pigmented tissue [91, 92].

Genetic variations of angiogenic factors have important consequences for the clinical treatment of angiogenesis-dependent diseases. For example, some tumours that appear poorly vascularized and have a low microvessel density will be inhibited by a significantly lower dose of an angiogenesis inhibitor than is required for a highly vascularized tumour with a significantly higher microvessel density[94] This is counterintuitive to clinicians who may inform a patient that their tumour, "is not very vascular and therefore will not respond to antiangiogenic therapy." In fact, these tumours may be expressing their own angiogenesis inhibitors[95,96] and may respond to a lower therapeutic dose of angiogenesis inhibitor than would be required for a highly vascularized tumour.

The genetic heterogeneity of the angiogenic response is another reason for the pressing need to develop blood or urine biomarkers[97] in order to optimize the dosing of antiangiogenic therapy. Furthermore, when mice are used for pre-clinical studies of angiogenesis inhibitors, it is critical to know the genetic background of mice relative to their angiogenic responsiveness.

Optimising the application of antiangiogenic therapy

Insights into the molecular mechanisms and significance of angiogenesis in different biological contexts are creating exciting new opportunities for drug discovery. However, as in some cases, notably cancer, antiangiogenic therapies may also be used in combination with existing drugs, it is important to understand the difference between antiangiogenic and cytotoxic drugs in order to optimise efficacy.

Antiangiogenic therapy and cytotoxic chemotherapy

In February, 2004, when the FDA approved bevacizumab for colorectal cancer, Mark McClellan, the FDA commissioner said, "Anti-angiogenic therapy can now be considered the fourth modality for cancer treatment."[98]. It is a different modality because there are

certain notable differences about chemotherapy that do not always readily transfer to antiangiogenic therapy.

Importantly, antiangiogenic therapy primarily targets the activated microvascular endothelial cell in a tumour bed rather than the tumour itself. It may accomplish this directly by preventing endothelial cells from responding to angiogenic proteins, as endostatin[28] and Caplostatin[116,117] do. Antiangiogenic therapy can also inhibit endothelial cell proliferation and motility indirectly by suppressing a tumour's production of angiogenic proteins, as erlotinib does, or by neutralizing one of these proteins, as Bevacizumab does. For example, erlotinib decreases VEGF expression by both hypoxia-inducible factor (HIF)-1-alpha-independent and HIF-1-dependent mechanisms[139].

Also, while chemotherapy is usually more effective on rapidly growing tumours than on slowly growing tumours, the opposite is often true of antiangiogenic therapy. More rapidly growing tumours can require higher doses of antiangiogenic therapy[94]. Further, chemotherapy is optimally given at maximum tolerated dose, with off-therapy intervals of 1 to 3 weeks to rescue bone marrow and intestine. Antiangiogenic therapy may optimally require that endothelial cells be exposed to steady blood levels of the inhibitor[96]. Therefore, daily dosing is optimal for those angiogenesis inhibitors with a short half-life. However, certain antibodies such as bevacizumab can be administered every 2 weeks because of long-lasting antibody levels in plasma, and perhaps because of neutralization of VEGF in platelets by bevacizumab that enters the platelets and binds with VEGF[99]**.** Zoledronate (Zometa) is an amino-bisphosphonate that has been shown to inhibit angiogenesis,[100] by targeting MMP-9 [100a], by reducing circulating levels of pro-angiogenic proteins in the circulation[100b], or by suppressing multiple circulating pro-angiogenic factors in cancer patients [100c]. It accumulates in bone and can therefore be administered every month. However, after prolonged use, zoledronate may need to be administered less frequently to avoid osteonecrosis of the jaw. **[AU:ok?] [AU: OK]**

Another important difference concerns the side-effects of antiangiogenic therapy compared to chemotherapy. Bone marrow suppression, hair loss, severe vomiting and diarrhea, and weakness are less common with anti-angiogenic therapy and endostatin has shown minimal or no side-effects in animals[133] and in humans[134]. It has to be noted, though that certain angiogenesis inhibitors increase the incidence of thrombotic complications, for example thalidomide[101], and bevacizumab. The risk of thrombosis is increased when these angiogenesis inhibitors are administered together with conventional chemotherapy[102]. Other side-effects of inhibitors of VEGF include hypertension, intratumoural bleeding, and bowel perforation, especially in cases where the intestine is involved with tumour. Thalidomide has a slightly higher incidence of thromboembolic complications, as well as constipation and peripheral neuropathy—these are usually reversible upon discontinuation of thalidomide. Revlimid, an FDA approved derivative of thalidomide, has significantly reduced side-effects. Side-effects need to be carefully considered, especially when anti-angiogenic and cytotoxic medications are combined. To date there is almost no data that allows a direct comparison of clotting risk for anti-angiogenic therapy alone, compared to cytotoxic therapy/combination therapy.

However, there may also be unexpected benefits from combining angiogenesis inhibitors, or drugs that have varying degrees of anti-angiogenic activity with conventional chemotherapy **[AU:ok?] [AU:OK]**. For example, Jain has shown that bevacizumab, by decreasing vascular leakage in a tumour, can lower intratumuoural tissue pressure and increase delivery of chemotherapy to a tumour[136]. In other words, antiangiogenic therapy may "normalize" tumour vessels[137]. Teicher et al. earlier showed that antiangiogenic therapy could decrease intratumoural pressure which resulted temporarily in increased oxygenation to a tumour, with subsequent increased sensitivity to ionizing radiation[138].

Biphasic dose-efficacy of antiangiogenic therapy

Dose-efficacy is generally a linear function for chemotherapy. In contrast, several angiogenesis inhibitors have been reported to follow a biphasic, U-shaped dose-efficacy curve (known as 'hormesis'[110]). For example, interferon alpha is antiangiogenic at low doses, but not at higher doses[103], and Rosiglitazone, a PPAR gamma ligand, as well as endostatin protein therapy[105] (FIG. 3b) and endostatin gene therapy[106] inhibit angiogenesis with a U-shaped dose-efficacy curve[104]. In fact, before the U-shaped dose-efficacy response was recognized for antiangiogenic gene therapy, we had observed that gene therapy of endostatin could produce such high blood levels that all antiangiogenic activity was lost[107]**.** It is now clear that blood levels of certain angiogenesis inhibitors (such as endostatin), that are too high or too low will be ineffective, and that the biphasic dose-efficacy curve offers the best explanation for why endostatin gene therapy of murine leukemia failed[108,109]**.**

Even the effect of endostatin on the gene expression (for example HIF-1 alpha) of fresh human endothelial cells *in vitro* reveals a U-shaped dose-efficacy pattern[28]. This is important information for drug discovery. For example, in the Lucentis trial for age-related macular degeneration, a higher dose did not increase efficacy over a lower dose.

Antiangiogenic therapy and drug resistance

Tumours may become refractory to antiangiogenic therapy, especially if a mono-antiangiogenic therapy targets only one angiogenic protein (i.e. VEGF)[109]. Endothelial cells appear to have a lower probability of developing "resistance" to antiangiogenic therapy, even though mouse endothelial cells in a tumour bed can become genetically unstable[75,118]. While VEGF is expressed by up to 60% of human tumours, most tumours may also express 5 to 8 other angiogenic proteins - for example, human breast cancers can express up to 6 angiogenic proteins (FIG. 5). High-grade brain tumours may express more angiogenic proteins than other tumours. When the expression of one angiogenic protein is suppressed for a long period, the expression of other angiogenic proteins may emerge[111]. The mechanism of this "compensatory" response is unclear. Some angiogenesis inhibitors target up to three angiogenic proteins, while others target a broad spectrum of angiogenic proteins (TABLE 6). Certain tumours, such as high grade giant cell tumours and angioblastomas, produce bFGF as their predominant angiogenic protein and do not seem to deviate from this. For this reason, low dose daily interferon alpha therapy **[AU: please insert brief sentence to explain how IFNα affects bFGF]** for 1 to 3 years is sufficient to return abnormally high levels of bFGF in the urine of these patients to normal. Interferon-alpha has been reported to suppress production of bFGF by human cancer cells.[103] This treatment regimen has produced long term complete remissions (up to 10 years), without drug resistance, at this writing[112–114] and (Leonard Kaban, Massachusetts General Hospital, personal communication).

Currently, the majority of FDA-approved angiogenesis inhibitors, as well as those in Phase III clinical trials, neutralize VEGF, target its receptor, or suppress its expression by tumour cells (FIGURE 6). When "drug resistance" develops to some of these inhibitors, they are often perceived to represent the whole class of angiogenesis inhibitors. It remains to be seen if broad-spectrum angiogenesis inhibitors will develop less "drug resistance" than angiogenesis inhibitors targeted against a single angiogenic protein. In experimental tumours, TNP-470, a synthetic analogue of fumagillin and Caplostatin, its derivative[115,116], did not induce drug resistance when administered to mice for prolonged periods of time[117].

Antiangiogenic chemotherapy (metronomic therapy)

Timothy Browder et al. first reported that when murine tumours were made drug resistant to cyclophosphamide, and cyclophosphamide was administered on a conventional chemotherapy maximum tolerated dose schedule, all mice died of large tumours[119]. However, if cyclophosphamide was administered more frequently at a lower dose, the tumours were potently inhibited because of endothelial apoptosis. If an angiogenesis inhibitor *(TNP-470, a fumagillin analogue*[117]*)* was added that by itself

could only inhibit the tumours by 50%, the drug resistant tumours were eradicated[119]. This experiment demonstrated a new principle: a cytotoxic chemotherapeutic agent could be redirected to an endothelial target by changing its dose and frequency of administration. Browder et al. called this regimen, "antiangiogenic chemotherapy." Klement and Kerbel confirmed this approach with a different chemotherapeutic agent[120]. Bocci *et al.*[121] further showed that antiangiogenic chemotherapy increased circulating thrombospondin-1, and that deletion of the thrombospondin-1 gene in mice completely abrogated the anti-tumour effect of this antiangiogenic therapy. This result suggested that thrombospondin-1 acts as a mediator of antiangiogenic chemotherapy[121]. The optimization of chemotherapy to treat vascular endothelium in the tumour bed is also called "metronomic" therapy[122] (FIG. 3c), and has entered clinical trials for brain tumours and other tumours refractory to conventional chemotherapy. Kieran et al. reported a recent study of 20 children with different types of brain tumours refractory to surgery, radiotherapy, and chemotherapy, who were treated for 6 months with daily oral thalidomide and celecoxib, plus daily low dose oral cyclophosphamide alternated every 21 days with daily low dose oral etoposide[123]**.** Twenty-five percent of the patients were progression free more than 2.5 years from starting therapy. Forty percent of patients completed the 6 months of therapy, resulting in prolonged or persistent disease-free status. Sixteen percent of patients showed a radiographic partial response. Only elevated thrombospondin-1 levels in the blood correlated with prolonged response. This is consistent with the elevated circulating thrombospondin-1 levels observed in tumour-bearing mice treated with antiangiogenic (metronomic) cyclophosphamide[121]. It is possible that angiogenesis inhibitors, such as bevacizumab, may be augmented by low dose antiangiogenic (metronomic) chemotherapy with fewer side effects than conventional dosing of chemotherapy.

New pharmacology: certain oral drugs may increase endogenous angiogenesis inhibitors

The clinical clue that individuals with Down syndrome have an elevated circulating level of endostatin approximately 1.6 fold higher than normal individuals[87] is provocative. It suggests that small elevations of one or more endogenous angiogenesis inhibitors in the blood could protect against recurrent cancer, or could prevent the switch to the angiogenic phenotype in women at high risk for breast cancer. It is also possible that other genes on chromosome 21 have antiangiogenic activity.

It has been found that certain orally available small molecules can upregulate expression of specific endogenous antiangiogenic proteins, opening the way for a new field of pharmacology (FIG.7). Endostatin is increased by Tamoxifen[124], celecoxib[125]**,** and (in joint fluid) by prednisolone plus salazosulfapyridine[126]. Thrombospondin-1 is upregulated by low dose cyclophosphamide[121], doxycycline[127] and by Rosiglitazone[104]. This unifying concept points to future drug discovery in which the 29 known endogenous angiogenesis inhibitors could be screened for small molecule inducers that would increase the circulating level of one or more of them.

Outlook

Angiogenesis inhibitors are now being approved and introduced into medical practice throughout the world. At the same time, a need for molecular biomarkers is being met by an expanding world-wide research effort to develop gene-based and protein-based molecular signatures in the blood, platelets, and urine, for very early diagnosis of recurrent cancer. One can speculate that if these two fields intersect, it may someday be possible to diagnose microscopic tumours at a millimeter size, at about the time of the angiogenic switch, but perhaps years before they are symptomatic, or before they can be "visualized" by any conventional methods.

For example, today most individuals with the diagnosis of colon cancer are operated upon. At least 50-60% of these patients are cured by the surgery. In the other patients, cancer will recur in approximately 4-6 years. Physicians are helpless to do anything until symptoms occur (e.g., pain, jaundice, etc), or until the recurrent cancer can be located by ultrasound, magnetic resonance imaging, or CAT scan. However, sensitive and specific molecular biomarkers being developed today could be used in the future to diagnose the presence of a microscopic recurrent tumour even before it could be anatomically located. Once these biomarkers are validated in clinical trials, then physicians could "treat the rising biomarker" with relatively non-toxic angiogenesis inhibitors, until the biomarker returned to normal. A paradigm shift would be that recurrent cancer would be treated without waiting to see it, when it is still relatively harmless with low or no metastatic potential (i.e., prior to the switch to the angiogenic phenotype). It may also be possible to use angiogenesis-based biomarkers to monitor the progression or regression of certain angiogenesis-dependent diseases that are non-neoplastic. These could include atherosclerosis, endometriosis, Crohn's disease and rheumatoid arthritis, among others.

There may be an analogy in the history of the treatment of infection. Before 1930, there were virtually no drugs for any infection, and most infections progressed to abscesses. Surgeons had to wait until the abscess was large enough to be located by X-rays so that the abscess could be surgically drained. The surgical textbooks of that era instructed surgeons how to locate an abscess: "above the liver, behind the liver, in the mastoid," etc. The term 'laudable' pus was commonly used to mean that if a surgeon could successfully drain an abscess, the patient might live. After 1941, when antibiotics were introduced, it was no longer necessary to precisely locate an infection. Today the treatment of most infections is simply guided by blood tests (white blood cell count, or blood cultures). As we continue to gain insight into angiogenesis and the role of angiogenic factors in seemingly unrelated diseases, the consequent potential of angiogenic modulators could see Peter Carmeliet's prediction in the December 2005 issue of Nature[128] becoming prophetic. He wrote, "Angiogenesis research will probably change the face of medicine in the next decades, with more than 500 million people worldwide predicted to benefit from pro- or anti-angiogenesis treatments"[128].

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Competing interests statement
The authors declare no competing financial interests.

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Figure 1| **Key steps in tumour angiogenesis.** Angiopoietin-1, expressed by many cells, binds to the endothelial Tie-2 receptor and helps maintain a normalized state in blood vessels. VEGF (vascular endothelial growth factor) is secreted by tumour cells and binds to its receptor (VEGFR-2) and to neuropilin on endothelial cells. It is the most common of at least 6 other pro-angiogenic proteins from tumours. Matrix metalloproteinases (MMPs) are released from tumour cells, but also by VEGF-stimulated endothelial cells. MMPs mobilize pro-angiogenic proteins from stroma, but can also cleave endostatin from collagen 18 in the vessel wall, and participate in the cleavage of angiostatin from circulating plasminogen.
Tumour cells secrete angiopoietin-2 which competes with angiopoietin-1 for binding to the endothelial Tie-2 receptor. Angiopoietin-2 increases degradation of vascular basement membrane and migration of endothelial cells, thus facilitating sprout formation. PDGF (platelet-derived growth factor), an

angiogenic protein secreted by some tumours, can upregulate its own receptor on endothelial cells. bFGF (basic fibroblast growth factor) is secreted by other tumours. Integrins on endothelial cells carry signals in both directions. Integrins facilitate endothelial cell binding to extracellular membranes, a requirement for the cells to maintain viability and responsiveness to growth regulatory proteins. Endothelial cells are among the most anchorage-dependent cells. Certain pro-angiogenic proteins upregulate endothelial integrins, thought to sustain endothelial cell viability during the intermittant detachments required to migrate toward a tumour, and to simultaneously increase their sensitivity to growth regulators (both mitogenic [VEGF or bFGF], and anti-mitogenic [endostatin]). New endothelial cells do not all originate from neighboring vessels. A few arrive as precursor bone marrow-derived endothelial cells.
Endothelial growth factors are not all delivered to the local endothelium directly from tumour cells. Some angiogenic regulatory proteins (both pro- and anti-angiogenic) are scavenged by platelets, stored in alpha granules and appear to be released within the tumour vasculature.

Figure 2| [AU:please provide figure title and legend, and a reference to this figure in the text]

Figure 3| **Examples of antiangiogenic therapy**
a: Phase III clinical trial of Lucentis, a fragment of an antibody to VEGF used for intraocular injection in patients with macular degeneration [30].
b: A biphasic (U-shaped) dose-efficacy curve for human pancreatic cancer in immunodeficient mice treated with endostatin. The tumor cells are also p53-deficient (from [90].
c: Dosing schedule differences between conventional chemotherapy and antiangiogenic (metronomic) chemotherapy (adapted from [99] and from discussions with Robert Kerbel, (Toronto).

Figure 4| **Oncogene-addiction is angiogenesis dependent.**
An oncogene-induced tumor that cannot recruit new blood vessels will remain as a harmless microscopic sized tumor in experimental animals [62].

Figure 5| **Angiogenic proteins in breast cancer**
Human breast cancer can express at least 6 different angiogenic proteins (adapted from [111]).

Figure 6| [AU:please provide figure title and legend]

Figure 7| **Small molecules to increase endogenous angiogenesis inhibitors**
Examples of small molecules that are orally available and may induce increased levels of endogenous angiogenesis inhibitors in the blood or joint fluid.

Figure 1.

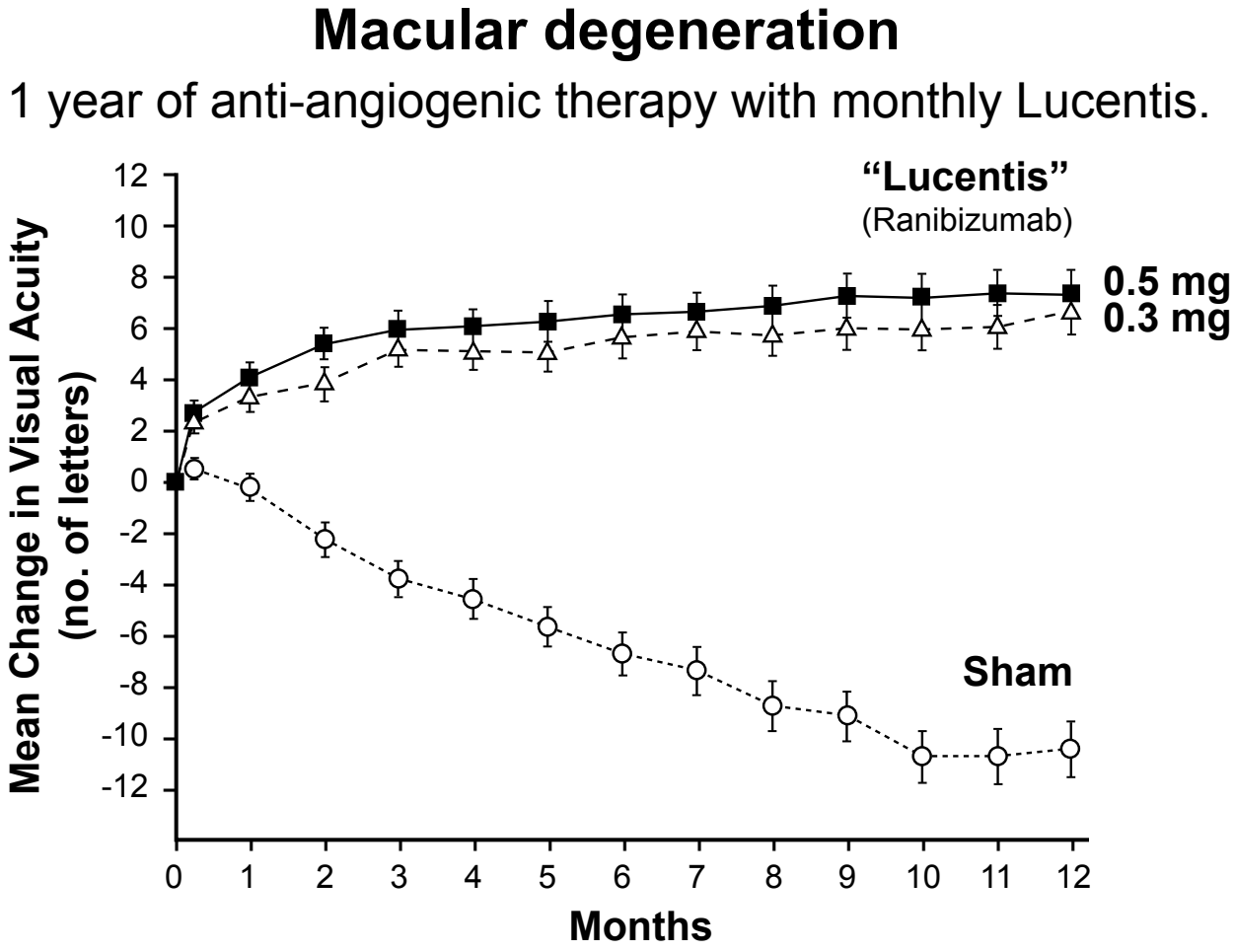

Macular degeneration
1 year of anti-angiogenic therapy with monthly Lucentis.
"Lucentis"
(Ranibizumab)
0.5 mg
0.3 mg
Sham
Mean Change in Visual Acuity
(no. of letters)
Months
12
10
8
6
4
2
0
-2
-4
-6
-8
-10
-12
0
1
2
3
4
5
6
7
8
9
10
11
12

Figure 2.

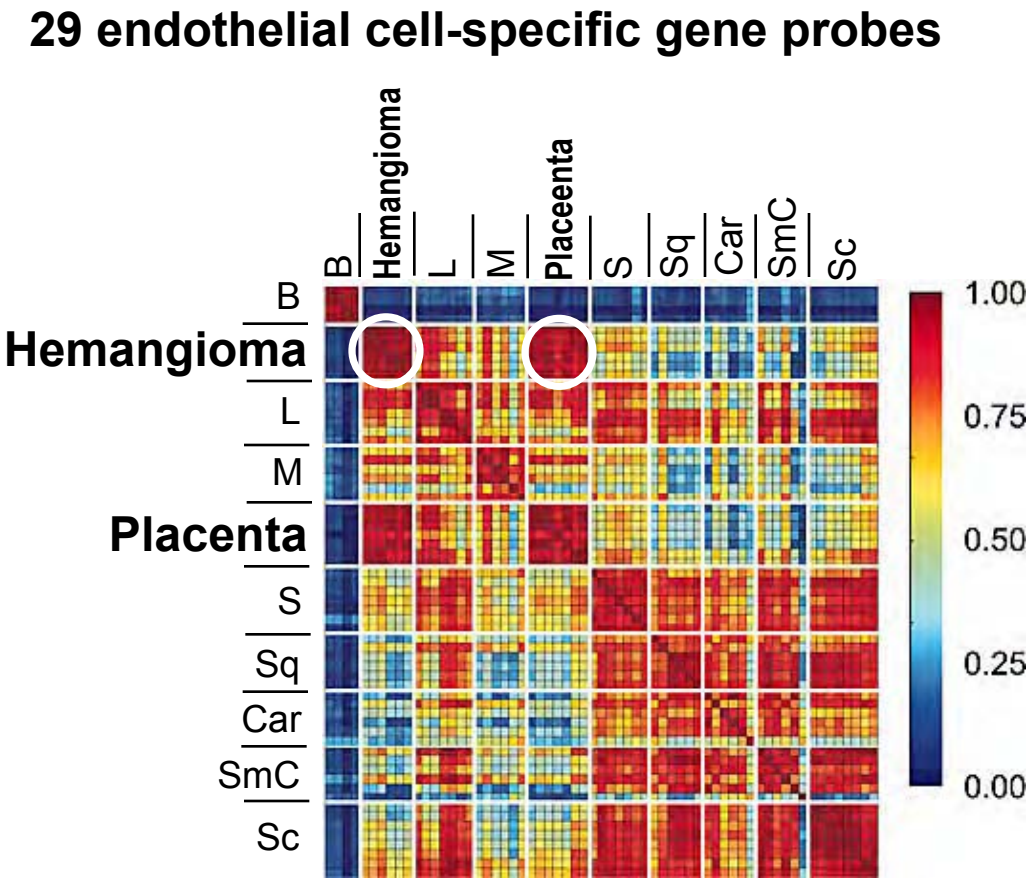
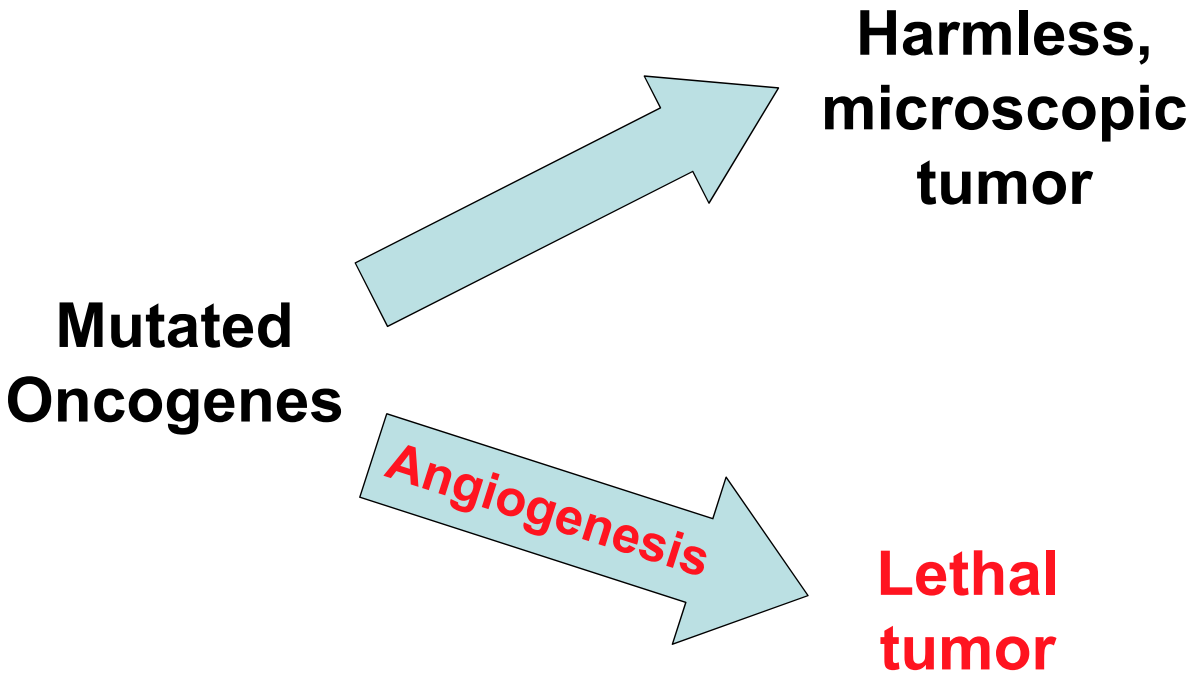

29 endothelial cell-specific gene probes
B
Hemangioma
L
M
Placenta
S
Sq
Car
SmC
Sc
1.00
0.75
0.50
0.25
0.00

Figure 3.



Oncogene-addiction is angiogenesis-dependent.

Figure 4.

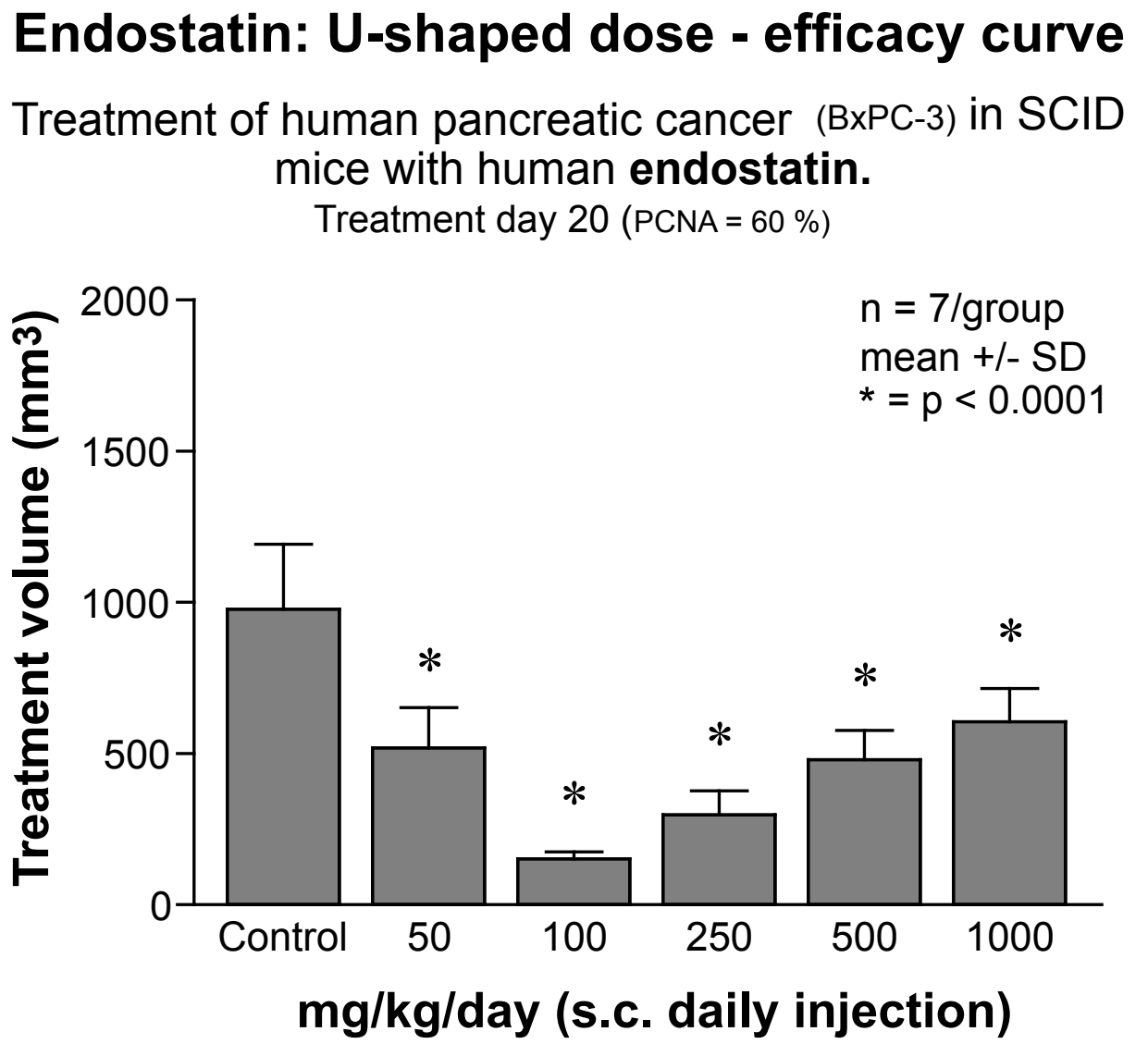

Endostatin: U-shaped dose - efficacy curve
Treatment of human pancreatic cancer (BxPC-3) in SCID mice with human endostatin.
Treatment day 20 (PCNA = 60 %)
n = 7/group
mean +/- SD
* = p < 0.0001
Treatment volume (mm3)
2000
1500
1000
500
0
Control
50
100
250
500
1000
mg/kg/day (s.c. daily injection)

Figure 5.

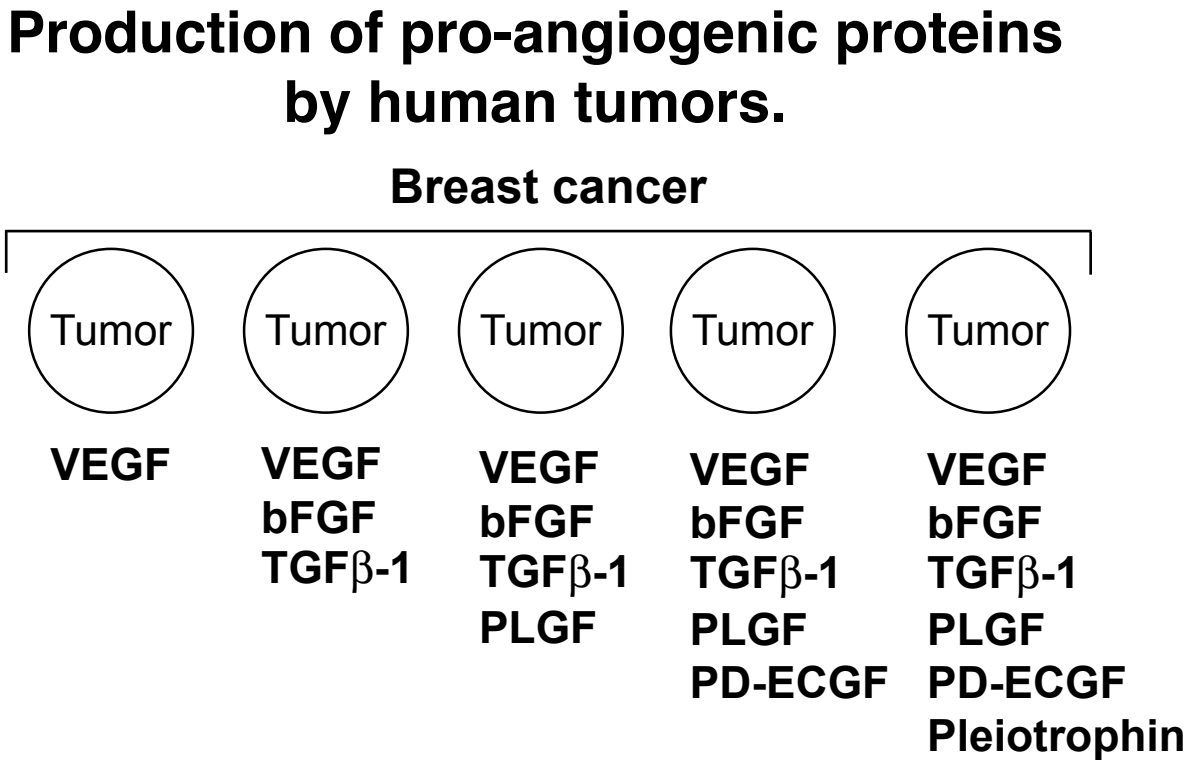

Production of pro-angiogenic proteins by human tumors.
Breast cancer
Tumor
Tumor
Tumor
Tumor
Tumor
VEGF
VEGF
bFGF
TGFβ-1
VEGF
bFGF
TGFβ-1
PLGF
VEGF
bFGF
TGFβ-1
PLGF
PD-ECGF
VEGF
bFGF
TGFβ-1
PLGF
PD-ECGF
Pleiotrophin

Figure 6.

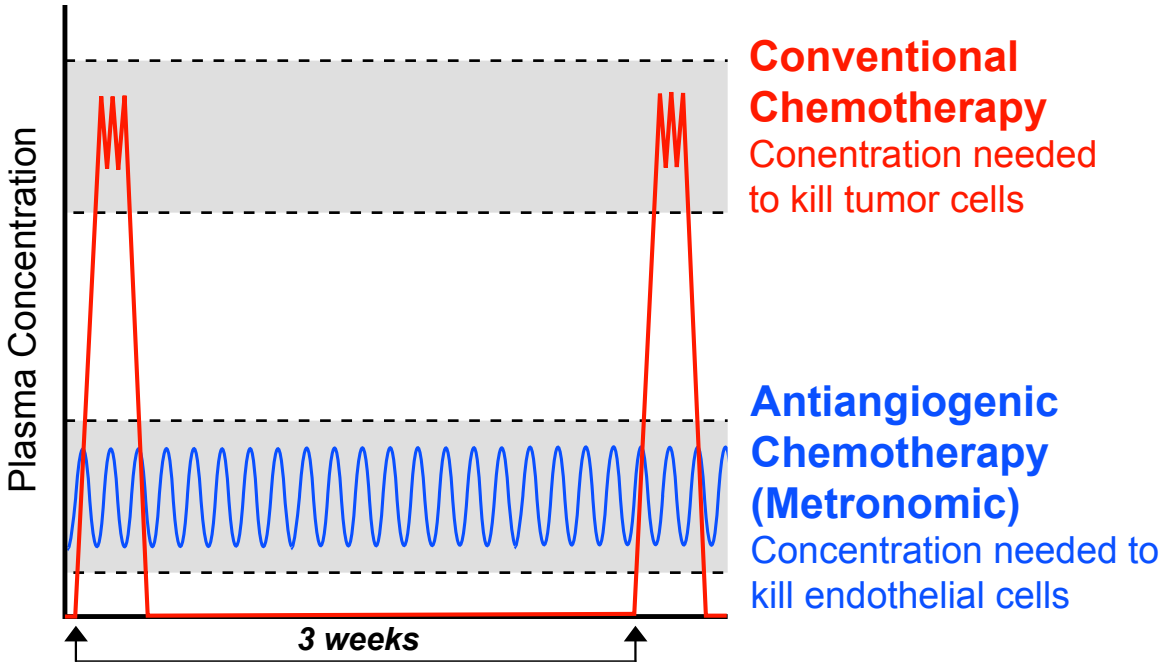

Plasma Concentration
Conventional Chemotherapy
Conentration needed to kill tumor cells
Antiangiogenic Chemotherapy (Metronomic)
Concentration needed to kill endothelial cells
3 weeks

Figure 7.

Orally administered small molecules which increase **endogenous** angiogenesis inhibitors**:** ***An emerging pharmaceutical field?***

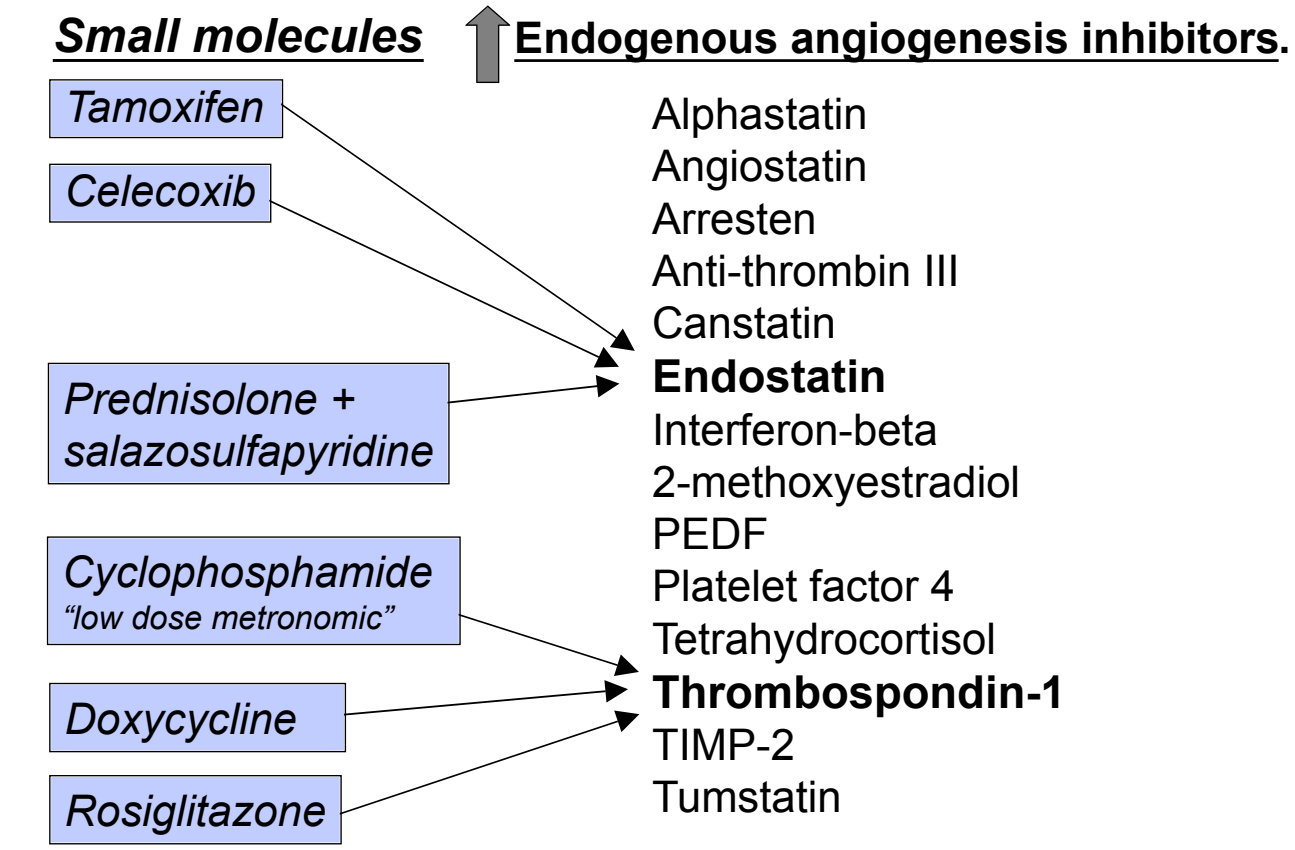
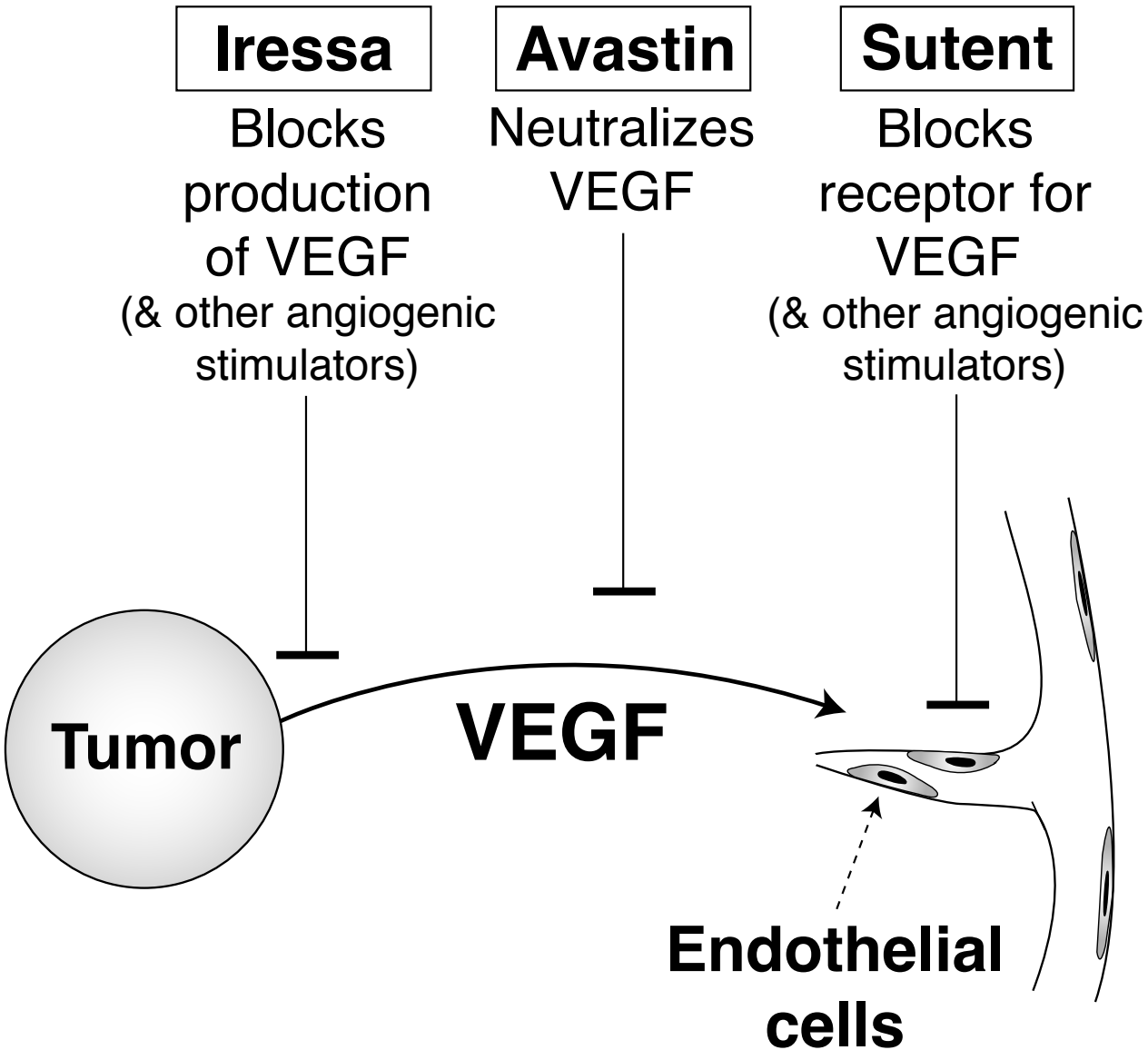


Figure 7.


Iressa
Blocks production of VEGF (& other angiogenic stimulators)
Avastin
Neutralizes VEGF
Sutent
Blocks receptor for VEGF (& other angiogenic stimulators)
Tumor
VEGF
Endothelial cells

In vivo vasculogenic potential of human blood-derived endothelial progenitor cells

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Short title: Vasculogenic potential of blood-derived EPCs

Keywords: vasculogenesis; blood vessels; TE; EPC

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Abstract

Vascularization of engineered tissues is one of the major challenges of tissue-engineering (TE). We hypothesize that blood-derived endothelial progenitor cells (EPCs) have the required proliferative and vasculogenic activity to create vascular networks *in vivo*. To test this, EPCs isolated from human umbilical cord blood or from adult peripheral blood, and human saphenous vein smooth muscle cells (HSVSMCs) as a source of perivascular cells, were combined in Matrigel and implanted subcutaneously into immunodeficient mice. Evaluation of the implants at one week revealed an extensive network of human specific lumenal structures containing erythrocytes, indicating the formation of functional anastomoses with the host vasculature. Quantitative analyses showed that the microvessel density was significantly superior to that generated by mature human dermal microvascular endothelial cells (HDMECs). We also found that as EPCs were expanded in culture, their morphology, growth kinetics and proliferative responses toward angiogenic factors progressively resembled those of HDMECs, indicating a process of *in vitro* cell maturation. This maturation correlated with a decrease of the degree of vascularization *in vivo*, which could be compensated by increasing the number of EPCs seeded into the implants. Our findings strongly support the use of human EPCs to form vascular networks in engineered organs and tissues.

INTRODUCTION

TE holds promise as a new approach for creating replacement tissue to repair congenital defects or diseased tissue[1]. One strategy is to seed the appropriate cells on a biodegradable scaffold engineered with the desired mechanical properties, followed by stimulation of cell growth and differentiation *in vitro*, such that, on implantation *in vivo*, the engineered construct undergoes remodeling and maturation into functional tissue [2]. Examples of this approach include blood vessels and cardiovascular substitutes, where autologous vascular cells have been used for this purpose without immune rejection [3-5]. Despite advances in this field, TE still faces important constraints. There are no TE constructs presently available that have an inherent microvascular bed ready to be connected to the host vascular system. Consequently, tissues implanted with a volume greater than 2 to 3 mm^3 cannot obtain appropriate provision of nutrients, gas exchange, and elimination of waste products since all these mechanisms are limited by the diffusion distance [6]. To overcome the problem of vascularization, strategies such as embedding angiogenic factors into the scaffold to promote ingrowth of microvessels, fabrication technologies to create polymers containing vessel-like networks, and pre-vascularization of matrices prior to cell seeding have been proposed [7-11].

The need for prefabricated channels or growth factor-induced angiogenesis could be avoided by exploiting the inherent vasculogenic ability of endothelial cells (ECs). Using human umbilical vein ECs (HUVECs), microvascular networks in collagen/fibronectin gels were formed within 31 days of implantation into immunodeficient mice [12]. Similar results have been reported with human microvascular ECs (HDMECs) seeded on biopolymer matrices, where functional microvessels were evident 7-10 days after implantation into mice [13]. Nevertheless, the clinical use of mature ECs derived from autologous vascular tissue presents some important limitations: 1) the isolation relies on an invasive procedure, 2) mature ECs cells show relatively low proliferative potential, and 3)

the difficulty of obtaining a sufficient number of cells from a small biopsy of autologous tissue. These limitations has instigated the search for other sources of ECs with more proliferative and vasculogenic activities such as those derived from both embryonic and adult stem and progenitor cells [14]. One recent example showed how seeding of endothelial cells derived from embryonic stem cells along with myoblast and embryonic fibroblasts resulted in the formation of skeletal muscle tissue[15]. However, ethical considerations along with a poor understanding of the mechanisms controlling the differentiation of embryonic stem cells are hurdles that need to be overcome before these cells can be used in a clinical setting.

For clinical applications, the presence of endothelial progenitor cells (EPCs) in circulation represents a promising opportunity to non-invasively obtain the required endothelial population [16]. In previous work we showed the creation of microvascular networks *in vitro* using biodegradable scaffolds seeded with EPCs that had been isolated from human umbilical cord blood and expanded *in vitro* as mature ECs [17]. Using a sheep model, we also showed that blood-derived EPCs could endothelialize small-diameter blood vessels [5]. We now propose that human blood-derived EPCs constitute a robust source of ECs with the potential to form functional capillary networks *in vivo*. To test this, we used a xenograft model where human cells were mixed in Matrigel and implanted subcutaneously into immunodeficient mice. Our goal was to advance feasibility studies by evaluating the ease with which highly purified and phenotypically defined human EPCs can create microvascular structures that form functional anastomoses with the host vasculature.

MATERIAL AND METHODS

Isolation and culture of blood-derived EPCs

Human umbilical cord blood was obtained from the Brigham and Women's Hospital in accordance with an Institutional Review Board-approved protocol. Adult peripheral blood was collected from volunteer donors in accordance with a protocol approved by Children's Hospital Boston Committee on Clinical Investigation. Both cord blood-derived EPCs (cbEPCs) and adult peripheral blood-derived EPCs were obtained from the mononuclear cell (MNC) fractions similarly to other authors [18-20]. MNCs were seeded on 1% gelatin-coated tissue culture plates using Endothelial Basal Medium (EBM-2) supplemented with SingleQuots (except for hydrocortisone) (Cambrex BioScience, Walkersville, MD), 20% FBS (Hyclone, Logan, UT), 1x glutamine-penicillin-streptomycin (GPS; Invitrogen, Carlsbad, CA) and 15% autologous plasma [17]. Unbound cells were removed at 48 hours for cord blood and at 4 days for adult blood. In both cases, the bound cell fraction was then maintained in culture using EBM-2 supplemented with 20% FBS, SingleQuots (except for hydrocortisone) and 1x GPS (this medium is referred to as EBM-2/20%). Colonies of endothelial-like cells were allowed to grow until confluence, trypsinized and purified using CD31-coated magnetic beads (Dynal Biotech, Brown Deer, WI) (see Supplemental Figures 1 and 5). CD31-selected EPCs were serially passaged and cultured on fibronectin-coated (FN; 1 ug/cm^2; Chemicon International, Temecula, CA) plates at 5x10^3 cell/cm^2 in EBM-2/20%. HDMECs from newborn foreskin cultured in the same condition as cbEPCs were used as positive controls [21]. Human saphenous vein smooth muscle cells (HSVSMCs) grown in DMEM (Invitrogen), 10% FBS, 1x GPS and 1x Non essential amino acids (Sigma-Aldrich, St. Louis, MO) were used as negative controls for endothelial phenotype.

Phenotypic characterization of cbEPCs

Methods for flow cytometry, indirect immunofluorescence and RT-PCR are described in the on-line Supplement.

In vitro maturation of cb EPCs

Expansion potential of cbEPCs

cbEPCs and adult EPCs, isolated as described above, were expanded for 112 and 60 days, respectively. All passages were performed by plating the cells onto 1 ug/cm^2 FN-coated tissue culture plates at 5x10^3 cell/cm^2 using EBM-2/20%. Medium was refreshed every 2-3 days and cells were harvested by trypsinization and re-plated in the same culture conditions for the next passage. Cumulative values of total cell number were calculated by counting the cells at the end of each passage using a haemocytometer.

Growth kinetics assay

Growth curves of cbEPCs were evaluated at different passages. Cells were plated in triplicates onto 1 ug/cm^2 FN-coated 24-well tissue culture plates at 5x10^3 cell/cm^2 in 0.5 ml of EBM-2/20%. Medium was refreshed every two days and cell numbers evaluated at 24 hour intervals for 7 days by counting the cells after trypsinization using a haemocytometer. Doubling time profiles were calculated from the mean values obtained from each growth curve at different passages [22].

Cell size measurements

Morphological differences of cbEPCs were evaluated at different passages. Confluent cell monolayers were immunostained with VE-cadherin antibody for cell surface and DAPI for nuclear visualization as described above. The areas occupied by cell bodies and cell nuclei were measured by analysis (ImageJ software, NIH) of the images obtained from randomly selected fields from three separate cultures after immunostaining. All values were normalized to the value of total cell area.

Proliferation assay

Cells were seeded in triplicates onto 1 ug/cm^2 FN-coated 24-well plates at 5x10^3 cell/cm^2 using EMB-2 supplemented with 5% FBS and 1x GPS (control medium); plating efficiency was determined at 24 hours, then cells were treated for 48 hours using control medium in the presence or absent of either 10 ng/ml of

VEGF-A (R&D Systems) or 1 ng/ml bFGF (Roche Applied Science, Indianapolis, IN). Cells were trypsinized and counted using a haemocytometer. Values were normalized to the cell numbers determined at 24 hours.

In vivo vasculogenesis experiments

Matrigel implantations

Unless otherwise indicated, $1.5x10^6$ EPCs were mixed with $0.375x10^6$ HSVSMCs (4:1 ratio) and resuspended in 200 ul of Phenol Red-free Matrigel (BD Bioscience, San Jose, CA) on ice. The mixture was implanted on the back of a six-week-old male athymic nu/nu mouse (Charles River Laboratories, Boston, MA) by subcutaneous injection using a 25-gauge needle. One implant was injected per mouse. Each experimental condition was performed with 4 mice.

Histology and immunohistochemistry

Matrigel implants were removed at one week after xenografting, fixed in 10% buffered formalin overnight, embedded in paraffin, and sectioned. Hematoxylin and eosin (H&E) stained 7 μm-thick sections were examined for the presence of lumenal structures containing red blood cells. For immunohistochemistry, 7-μm-thick sections were deparaffinized, blocked for 30 minutes in 5% horse serum, and incubated with human-specific CD31 monoclonal antibody (1:50, DakoCytomation), anti-human α-SMA (1:750, Sigma-Aldrich), or mouse IgG (DakoCytomation) for 1 hour at room temperature. Horseradish peroxidase-conjugated secondary antibody and 3,3'-diaminobenzidine (DAB) were used for detection. The sections were counterstained with hematoxilin and mounted using Permount (Fisher Scientific).

Microvessel density analysis

Microvessels were detected by the evaluation of H&E stained sections taken from the middle part of the implants. The full area of each individual section was evaluated. Microvessels were identified and counted as lumenal structures containing red blood cells. The area of each section was estimated by image

analysis. Microvessels density was calculated by dividing the total number of red blood cell-filled microvessels by the area of each section (expressed as vessels/mm^2). Values reported for each experimental condition correspond to the average values obtained from four individual animals.

Statistical analysis

The data were expressed as means ± SD. Where appropriate, data were analyzed by analysis of variance (ANOVA) followed by two-tailed Student's unpaired t-tests. *P* value < 0.05 was considered to indicate a statistically significant difference.

RESULTS

Phenotypic characterization of cbEPCs

We isolated EPCs from the MNC fraction of human umbilical cord blood samples (n=19) similarly to other authors [18,19]. Cord blood-derived endothelial colonies (identified by typical cobblestone morphology) emerged in culture after one week (see Supplemental Figure 1). The size, frequency, and time of appearance of these colonies varied as already reported [18] (data not shown). Endothelial colonies were left to grow in the original culture plates until confluence and purified thereafter (at passage 1) by selection of CD31-positive cells (Supplemental Figure 1). This procedure resulted in superior cell yields compared to our previous isolation protocol based on double selection of CD34+/CD133+ cells from the MNC fraction [17]. However, since CD31 is not a specific marker of EPCs and due to the heterogeneity of blood preparations, both phenotypical and functional characterization were performed. This was especially important considering that earlier studies have shown that some EPC colonies isolated from MNCs contain cells that express the hematopoietic-specific cell-

surface antigen CD45 [14,23,24] , raising questions about the cellular origin of circulating EPCs.

The endothelial phenotype of the isolated cbEPCs was confirmed by different methods. Flow cytometric analysis of cbEPCs showed remarkably uniform expression of EC markers CD34, VEGF-R2, CD146, CD31, vWF and CD105 (Figure 1a). In addition, cells were negative for mesenchymal marker CD90 and hematopoietic markers CD45 and CD14, confirming that the cells were not contaminated with either mesenchymal or hematopoietic cells. Additionally, RT-PCR analyses showed the expression of EC markers CD34, VEGF-R2, CD31, VE-cadherin, vWF and eNOS at the mRNA level (Figure 1b). Indirect immunofluorescent staining was performed to further examine the expression of EC markers. The results showed that cbEPCs expressed CD31, VE-cadherin and vWF (Figure 1c). Importantly, the localization of CD31 and VE-cadherin at the cell-cell borders and vWF in a punctuate pattern in the cytoplasm showed clear indications of EC properties.

In addition, we tested whether cbEPCs were able to up-regulate leukocyte adhesion molecules in response to the inflammatory cytokine TNF-α. The low-to-undetectable levels of E-selectin, ICAM-1 and VCAM-1 in the untreated cbEPC cultures were up-regulated upon 5 hour incubation with TNF-α (Figure 1d). This response to an inflammatory cytokine is characteristic of ECs and suggests that the use of cbEPC in the formation of microvascular vessels could also provide physiologic proinflammatory properties.

In summary, this combination of analyses provides a definitive demonstration that the cells isolated from umbilical cord blood were ECs and discards the possibility of hematopoietic/monocytic cells in the culture[20]. Based on the isolation methodology and the phenotypical characteristics, our isolated cells are similar to those referred to by other authors as late-EPCs or endothelial

outgrowth cells[19,24]. The characterization depicted in Figure 1 corresponded to cbEPCs at passage 6. However, a detailed characterization was performed at passages 4, 9, 12 and 15 with similar results (Supplemental Figure 2, panels a-d), indicating a stable endothelial phenotype through long term culture. Furthermore, we provide additional characterization of the cbEPCs at passage 6 (Supplemental Figure 3) to show that cbEPCs express two other VEGF-receptors, neuropilin-1 and Flt-1 (panel a), and that the cbEPCs do not express the smooth muscle/mesenchymal cell markers PDGF-Rβ (panel b), α-SMA, or calponin (panel c).

In vivo vasculogenic potential of cbEPCs

Our previous work showed the creation of microvascular network *in vitro* by culturing cbEPCs and HSVSMCs on biodegradable scaffolds [17]. To answer the question of whether cbEPCs were capable of forming functional capillary networks *in vivo*, we implanted cbEPCs in Matrigel subcutaneously into nude mice for one week. For this experiment, $1.5x10^6$ of cbEPCs (passage 6) were combined with $0.375x10^6$ HSVSMCs in 200 ul of Matrigel, resulting in a ratio of cbEPCs to HSVSMCs of 4 to 1, and injected subcutaneously. This ratio of cbEPCs to HSVSMCs was less than the 1:1 ratio previously used [17], with the intention to minimize the contribution of smooth muscle cells. After harvesting the Matrigel implants, H&E staining revealed the presence of lumenal structures containing murine erythrocytes throughout the implants (Figure 2a). Similar results were obtained with cbEPCs isolated from three different cord blood samples. Importantly, implants with either cbEPCs or HSVMSCs alone failed to form any detectable microvessels after one week (Figures 2b and 2c). Injections of Matrigel alone resulted in the appearance of few host cells infiltrated into the borders of the implants (Figure 2d), indicating that Matrigel itself was not responsible for the presence of vascular structures within the implants.

To further characterize the microvascular structures detected, sections of the implant were immunohistochemically stained using a human-specific CD31

antibody. As depicted in Figure 2k, nearly all of the lumenal structures stained positive for human CD31, confirming that those lumens were formed by the implanted human cbEPCs and not by the host cells. This result was important because it demonstrated that the formation of microvascular vessels within the implant is the result of a process of in vivo vasculogenesis carried out by the implanted cells and it is not due to blood vessel invasion and sprouting, i.e., an angiogenic response from nearby host vasculature. The specificity of the anti-human CD31 antibody[25,26] was confirmed by the negative reaction obtained when mouse lung tissue sections were stained in parallel (Supplemental Figure 4). Taken together, the human endothelial identity of the lumenal structures (Figure 2k) and the presence of murine erythrocytes within those structures (Figure 2a,2h and 2j), it was evident that vasculogenesis occurred and, in addition, the newly created microvessels formed functional anastomoses with the host circulatory system. Next, the time course of vasculogenesis in the Matrigel was analyzed by harvesting implants at 2, 4 and 7 days after xenografting. At 2 days, a low degree of cellular organization was seen (Figure 2e). At 4 days, a high degree of organization with clear alignment of cells throughout the implant was observed, suggesting formation of cellular cords (Figure 2f). The presence of functional microvascular vessels, defined by the presence of red blood cells within the lumen, was appreciable one week after implantation (Figure 2g, h and j).

The location of the HSVSMCs was also examined by immunohistochemical staining using anti-α-SMA. Smooth muscle cells were detected both around the lumenal structures and throughout the Matrigel implants (Figure 2l), suggesting an ongoing process of vessel maturation and stabilization [27-29]. However, the α-SMA antibody is not human-specific, as shown by the positive staining of control tissue sections obtained from mouse lung (Supplemental Figure 4). Therefore, the observed α-SMA positive cells could corresponded to the implanted HSVSMCs or murine cells recruited from the host, or a combination of these.

Maturation of cbEPC during in vitro expansion

cbEPCs were serially passaged to determine their expansion potential. Remarkably, 10^{14} cells could theoretically be obtained after only 40 days in culture, and thereafter cells were expanded up to 70 population doublings (Figure 3a), which is consistent with previous studies [18]. Significant expansion of adult blood EPCs (10^8 cells) was also achieved under the same conditions using 50 milliliters of adult peripheral blood (Figure 3a). In addition to this enormous proliferative capacity, cbEPCs expressed and maintained a definitive endothelial phenotype *in vitro* as shown in Figure 1. However, neither the expansion potential nor the phenotypical stability rules out the possibility of cbEPCs undergoing cellular changes during their expansion *in vitro*. To investigate potential changes, the growth kinetics of cbEPCs at different passages were examined by the generation of growth curves (Figure 3b). We found that cells from earlier passages presented superior growth kinetics and reached higher cell densities at confluence. The former was confirmed by the generation of the doubling time profiles (Figure 3c), where lower passage number corresponded with shorter doubling times. The u-shape of these profiles is the result of mechanisms controlling cell growth *in vitro*: longer doubling times were found during both the early and late stages of the culture corresponding to the initial lag phase and the inhibition of cell growth by cell-cell contacts, respectively. Taking the minimum values as representative of the dividing capacity, cbEPCs presented minimum doubling times of 14, 17, 18, 29 and 35 hours at passages 4, 6, 9, 12, and 15 respectively. These results illustrated the remarkable dividing capacity of cbEPCs at low passage numbers, and showed that as cbEPCs were expanded in vitro, their growth kinetics progressively slowed.

Serially passaging of cbEPCs also resulted in evident morphological differences. As they were expanded, cells progressively occupied larger areas in culture (Figure 3d). While the areas occupied by the cell nuclei remained constant at

each passage, cbEPCs were found to be significantly ($P < .05$) smaller than the control HDMECs, with the exception of passage 15. As cbEPCs were expanded *in vitro*, the average area occupied by the cells increased towards that of HDMECs. The mean area of cbEPCs ranged from values 75% smaller than HDMECs at passage 4 to 17% smaller at passage 15. These results were consistent with the differences found in cell density at confluence (Figure 3b).

We next compared the proliferative response of cbEPCs at different passages to stimulation by angiogenic factors VEGF or bFGF (Figure 4). We found that both angiogenic factors produced a proliferative response in all the cases evaluated as compared to basal proliferation in the presence of 5% serum (control). The response was statistically significant ($P < .05$) in all the groups treated with bFGF. Interestingly, the proliferative response to bFGF was progressively reduced as passage number increased, and ranged from 5.4-fold at passage 4 to 2-fold at passage 15. When compared to HDMECs, the response toward bFGF was found significantly higher in cbEPCs at passages 4, 6 and 9, but not in the later passages. In the case of VEGF treatment, the response was statistically significant ($P < .05$) at passages 4 and 6 as compared to basal proliferation. Again, the proliferative response was progressively reduced as passage number increased, and varied from 3.1-fold in the earliest passage to 1.3-fold in the latest passage group. Collectively, these *in vitro* experiments demonstrate that despite the consistent and stable expression of endothelial markers, cbEPCs undergo cellular and functional changes as they are expanded in culture. Their morphology, growth kinetics and proliferative responses toward angiogenic growth factors progressively resembled those of HDMECs, indicating a process of *in vitro* cell maturation over time. We showed previously that proliferative responses of HDMECs isolated in our laboratory do not change from passage 3-12[21].

Effect of *in vitro* expansion of cbEPCs on *in vivo* vasculogenesis

We next tested whether the maturation of cbEPCs observed during their expansion *in vitro* has any effect on their vasculogenic ability *in vivo*. To answer this question, cbEPCs at different passages (3, 6, and 12) were implanted subcutaneously into nude mice in the presence of HSVSMCs. Examination after one week of the H&E-stained implants (Figure 5a-d) revealed a difference in the level of *in vivo* vascularization. Quantification of the red blood cell-containing microvessels (Figure 5e) showed that the differences among the groups were statistically significant ($P < .05$) in all the cases, with values ranging from 93 ± 18 vessels/mm^2 when using cbEPCs at passage 3 to 11 ± 13 vessels/mm^2 with passage 12. These results show that expansion of the cell population *in vitro* has indeed a significant impact in the subsequent performance *in vivo*. Parallel evaluation using mature HDMECs also revealed the presence of 23 ± 19 vessels /mm^2. This number of microvessels was inferior to those generated by the earliest passages of cbEPCs (passages 3 and 6), with values significantly higher in the case of cbEPCs at passage 3.

We tested whether the lower vasculogenic ability observed in expanded cbEPCs could be compensated by increasing the initial number of EPCs seeded into the implants. To evaluate this, we implanted either 0.5x10^6 (referred to as x1/3), 1.5x10^6 (x1) or 4.5x10^6 (x3) cbEPCs at passages 6 and 12 (Figure 6) in the presence of HSVSMCs at a constant 4:1 ratio. One week after xenografting, examination of the H&E-stained implants (Figure 6a-f) revealed that an increase in the number of cbEPCs resulted in a higher degree of *in vivo* vascularization. Quantification of the microvessel densities (Figure 6j) showed that the differences among the groups of cbEPCs at passage 6 were statistically significant ($P < .05$), with values ranging from 6 ± 7 vessels/mm^2 to 117 ± 23 vessels/mm^2 when using x1/3 or x3 respectively. Consistent with the previous results (Figure 5), the values of microvessel density in implants of cbEPCs at passage 6 were always higher than those at passage 12 when the same numbers of cbEPCs were used; indeed no microvessels were detected with x1/3

passage 12 cells. Nevertheless, at passage 12, the partial loss of vasculogenic potential was compensated by increasing the number of seeded cells. As seen in Figure 6, panels d-f and j, by simply seeding the implants with 3 times higher density of cbEPCs at passage 12, microvessel density was raised from 10 ± 6 vessels/mm^2 (x1) to 46 ± 28 vessels/mm^2 (x3). Furthermore, the microvessel level achieved with x3 cells passage 12 cells was similar to the level achieved with passage 6 cells at x1 (P=0.56).

We evaluated whether a similar approach (i.e., increasing the number of EPCs seeded) would result in increased vasculogenesis when using EPCs isolated from blood of adult volunteers (Supplemental Figure 5). To test this, we implanted either 1.5x10^6 (x1) or 4.5x10^6 (x3) adult EPCs at passages 6 in the presence of HSVSMCs (4:1 ratio). One week after xenografting, examination of the H&E-stained sections (Figure 6h and 6i) and human CD31-specific immunostaining (Figure 6g) revealed the presence of human microvessels containing red blood cells in both cases. As occurred with cbEPCs, we found that an increase in the number of adult EPCs resulted in a higher degree of *in vivo* vascularization with values ranging from 8 ± 8 lumens/mm^2 to 23 ± 4 lumens/mm^2 when using x1 or x3 adult EPCs respectively. Quantification of the microvessel densities (Figure 6j) showed that adult EPCs at x3 was similar to cbEPC-P6 x1 (P=0.10) and cbEPC-P12 x3 (P=0.2). In summary, these *in vivo* experiments clearly show that in addition to the cellular and functional changes observed *in vitro*, the vasculogenic ability of expanded EPCs progressively diminished but that this effect can be compensated by increasing the number of EPCs initially seeded in Matrigel.

DISCUSSION

Tissue vascularization is one of the major challenges to be addressed for the therapeutic success of TE applications. Here, we show that blood-derived EPCs

have an inherent vasculogenic ability that can be exploited to create functional microvascular networks *in vivo*. Implantation of EPCs with HSVSMCs resulted in the formation of an extensive blood vessel network after one week. The presence of human EC-lined lumens containing murine erythrocytes throughout the implants indicated not only a process of vasculogenesis by the implanted cells, but also the formation of functional anastomoses with the host circulatory system. Our results are the first to demonstrate the in vivo vasculogenic potential of blood-derived EPCs in a TE setting. Based on our results, we speculate that microvascular networks could be formed in many types of TE constructs with use of blood-derived EPCs.

Previous investigators have suggested the possibility of creating microvascular networks using mature ECs derived from vascular tissue. Both HUVECs and HDMECs, seeded into collagen/fibronectin gels and biopolymer matrices respectively, were shown to form complex vascular structures perfused by the host circulation after implantation into immunodeficient mice [12,13]. Alternatively, the use of fat-derived vessel fragments embedded into collagen gels was shown to generate perfused microvessels in immunodeficient mice [30]. Nevertheless, the necessity of invasive procedures together with their limited proliferative and vasculogenic ability represents important constraints for the clinical use of mature ECs derived from autologous vascular tissue.

For therapeutic applications, one critical requisite will be to isolate defined populations of cells so that growth and differentiation can be controlled and regulated during tissue development. Since first identified [16], EPCs have been isolated from the MNC fraction of blood in numerous studies [5,18-20,24,31,32]. However, their phenotypical characterization has been often controversial[20]. In our study, we show that 10^{11} homogeneous EPCs can be obtained from 25-50 ml of cord blood after 30 days in culture and 10^{8} EPCs from 50 ml of adult peripheral blood. These cell numbers are likely to exceed, in the case of cord

blood, and be sufficient, in the case of adult blood, what would be needed for most TE applications. Hence, we foresee that adult blood will also be a feasible source of EPCs for autologous TE and regenerative therapies. In summary, this remarkable yield confirms peripheral blood as a robust source of EC for autologous TE. Furthermore, our data demonstrate that these cells maintain both the expression of endothelial markers and functions through prolonged periods in culture.

As we previously demonstrated *in vitro* [17], the presence of vascular smooth muscle cells in the implants was found to be critical. Seeding cbEPCs with HSVSMCs dramatically enhanced the assembly of CD31+ cells into microvessel structures. Although the mechanisms by which the implanted smooth muscle cells facilitate the formation of vascular structures need to be further investigated, this finding is consistent with the extensive literature on endothelium-smooth muscle cells interactions in vascular development [27-29]. In our implants, α-SMA positive cells were detected both around the lumenal structures and throughout the Matrigel, suggesting an ongoing process of vessel assembly, maturation and stabilization [27,28]. We are currently investigating alternative sources that could provide autologous smooth muscle cells without the necessity of invasive procedures.

For TE applications, it is of most interest to determine the time course and the sequence of events that leads to the formation of functional microvessels. Time course analyses of the implants revealed that cells appeared dispersed throughout the implant by Day 2. Thereafter, cells became organized into tubular structures without red blood cells by Day 4, and formed functional erythrocyte-filled microvessels by Day 7. Hence, our *in vivo* model of tissue vascularization is well-suited for the study of the physiology of microvessel development and for the investigation of strategies to accelerate neovascularization. We speculate that, in Matrigel, these vessels would regress after some period of time due to

the lack of metabolic demand. In future studies, the usefulness of this approach for tissue vascularization will be tested by incorporating EPCs into tissue or organ constructs that requires a blood supply.

Another important finding of our study is related to the consequences the *in vitro* expansion had on vasculogenic ability. In previous work, we reported that far from remaining constant, the migratory capacity of EPCs *in vitro* decreased over time in culture [33]. Consistent with this concept, we have now shown that as cbEPCs were expanded in culture, their morphology, growth kinetics and proliferative responses toward angiogenic growth factors progressively resembled those of HDMECs, indicating a process of *in vitro* cell maturation over time. In addition, this maturation correlated with a decrease in the degree of vascularization *in vivo* (Figure 5). Even though at first examination this finding may seem to impose a limitation on the extent to which cbEPCs could be expanded *in vitro* prior to implantation, we showed that the partial loss of vasculogenic ability can be compensated by increasing the number of EPCs seeded into the implants (Figure 6). The number of EPCs in adult blood is known to be significantly lower than in cord blood [34], which implies a more extensive expansion in vitro will be needed to obtain a sufficient number. In addition, adult EPCs are also known to have an inherent lower proliferative capacity *in vitro* [18], which agrees with their lower vasculogenic ability *in vivo* reported here. Nevertheless, as occurred with cbEPCs after extensive expansion, these apparent limitations were overcome by increasing the number of adult EPCs seeded into the implants (Figure 6). Therefore, we speculate that the *in vivo* vasculogenic ability of EPCs from cord blood or adult peripheral blood can be modulated to the desired degree of vascularization.

In summary, our results strongly support the therapeutic potential of using human EPCs to form vascular networks that will allow sufficient vascularization of engineered organs and tissues. Further efforts are required to implement

strategies for controlled vasculogenesis in tissue engineered constructs using both autologous vascular endothelial and smooth muscle cells obtained from adult blood.

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Author contributions: Juan Melero-Martin designed, executed and interpreted all experiments and wrote the first draft of the manuscript. Zia A. Khan performed RT-PCR analyses and provided intellectual advice and assistance with all aspects of the animal studies. Arnaud Picard contributed intellectual advice and technical assistance with mouse studies. Xiao Wu provided invaluable expertise in the early stages of isolating cord blood cells. Sailaja Paruchuri performed analyses of adhesion molecule expression. Joyce Bischoff was involved in conceptual design of this project, interpretation of experimental results, and writing and editing drafts of the manuscript and figures.

FIGURES LEGENDS

Figure 1. Phenotypic characterization of EPCs. CD31-selected cbEPCs were evaluated at passage 6. HDMECs and HSVSMCs served as positive and negative controls respectively. (a) Cytometric analysis of cultured cbEPCs for endothelial markers CD34, VEGF-R2, CD146, CD31, vWF and CD105, the mesenchymal marker CD90, and hematopoietic/monocytic markers CD45 and CD14. Solid gray histograms represent cells stained with fluorescent antibodies. Isotype-matched controls are overlaid in a black line on each histogram. (b) RT-PCR analysis of cbEPCs for endothelial markers CD31, CD34, VEGF-R2, VE-cadherin, vWF and eNOS. (c) Indirect immunofluorescence of cultured cbEPCs grown in confluent monolayer showing positive staining for CD31, VE-cadherin, and vWF. (d) Up-regulation of E-selectin, ICAM-1 and VCAM-1 in cultured cbEPC in response to TNF-α. Solid gray histograms represent cells stained with fluorescent antibodies while black lines correspond to the isotype-matched control fluorescent antibodies.

Figure 2. In vivo vasculogenic potential of EPCs. Matrigel implants containing cbEPCs and/or HSVSMCs evaluated after one week. (a) H&E staining of implants (x100) containing a combination of cbEPCs (passage 6) and HSVSMCs, (b) only cbEPCs, (c) only HSVSMCs, (d) and Matrigel alone. H&E staining of implants containing both cbEPCs and HSVSMCs evaluated at day 2 (e), day 4 (f), and day 7 (g, x100; h, x400) after xenografting. (i-i) Matrigel plug containing cbEPCs and HSVSMCs harvested one week after implantation. (i) Macroscopic view of explanted Matrigel plug, (j) H&E staining showing high power view of one microvessel containing hematopoietic cells. (k) Immunohistochemical staining at one week with human specific CD31 antibody (x400) and with (l) α-SMA antibody. All images are representative of implants harvested from four different animals. (Black scale bar, 250 um; white scale bar, 50 um).

Figure 3. Growth kinetics and in vitro expansion of EPCs. (a) In vitro expansion of cbEPCs and adult blood EPCs isolated from mononuclear cells and purified by CD31-positive selection. (b) Growth curves of cbEPCs at different passage numbers (P4, P6, P9, P12 and P15). Each data point represents the mean of three separate cultures ± SD. (c) Doubling time profiles of cbEPCs at different passage numbers. Values were calculated from the mean values of cell number obtained at specific time points after plating. (d) Morphological differences of cbEPCs at increasing passage. Each bar represents the mean area ± SD obtained from randomly selected fields. All values were normalized to the total cell area occupied by HDMECs. * $P < .05$ compared to HDMECs.

Figure 4. Proliferative response toward angiogenic factors of EPCs. cbEPCs at different passage numbers (P4, P6, P9, P12 and P15) were seeded on FN-coated plates in EMB-2 supplemented with 5% FBS (control medium). After the initial 24 hour period, cells were treated with control medium in the presence or absent of either 10 ng/ml of VEGF or 1 ng/ml bFGF and assayed for cell number after 48 hours. Each bar represents the mean of three separate cultures ± SD, with values normalized to the values of cell density obtained at 24 hours when treatment began. * $P < .05$ compared to control. † $P < .05$ compared to equivalent treatment on HDMECs.

Figure 5. Effect of in vitro expansion of EPCs on in vivo vasculogenesis. Matrigel implants containing cbEPCs and HSVSMCs (4:1 ratio) were evaluated after one week. (a-c) H&E staining of Matrigel implants (x400) containing cbEPCs at passages 3 (a), 6 (b) and 12 (c). HDMECs implants (with HSVSMCs; 4:1 ratio) were used as control for mature ECs (d; x400). All images are representative of implants harvested from four different animals (Scale bar, 50 m). (e) Microvessel density in Matrigel implants was quantified by counting lumenal structures containing red blood cells. Each bar represents the mean microvessel density value determined from four separated implants and animals

± SD. * *P* < .05 compared to HDMEC. † *P* < .05 compared to cbEPC-P3. ‡ *P* < .05 compared to cbEPC-P6.

Figure 6. Effect of implanted cell number on vasculogenic performance of EPCs. Matrigel implants containing either $0.5x10^6$ (x1/3) (panels a, d), $1.5x10^6$ (x1) (panels b, e, h) or $4.5x10^6$ (x3) (panels c, f, i) EPCs in the presence of HSVSMCs (4:1 EPCs/HSVMSCs ratio) were evaluated after one week. (a-c) H&E staining of Matrigel implants containing cbEPCs at passages 6 (x400); (d-f) cbEPCs at passage 12 (x400) and (g-i) adult EPCs at passage 6 (x400). (g) anti-human CD31 immunostained section of adult EPCs at passage 6 seeded at x3. All images are representative of implants harvested from four different animals (Scale bar, 50 um). (j) Microvessel density was quantitated by counting lumenal structures containing red blood cells. Each bar represents the mean microvessel density value determined from four separated implants and animals ± SD. * *P* < .05 compared to x1/3. † *P* < .05 compared to x1.

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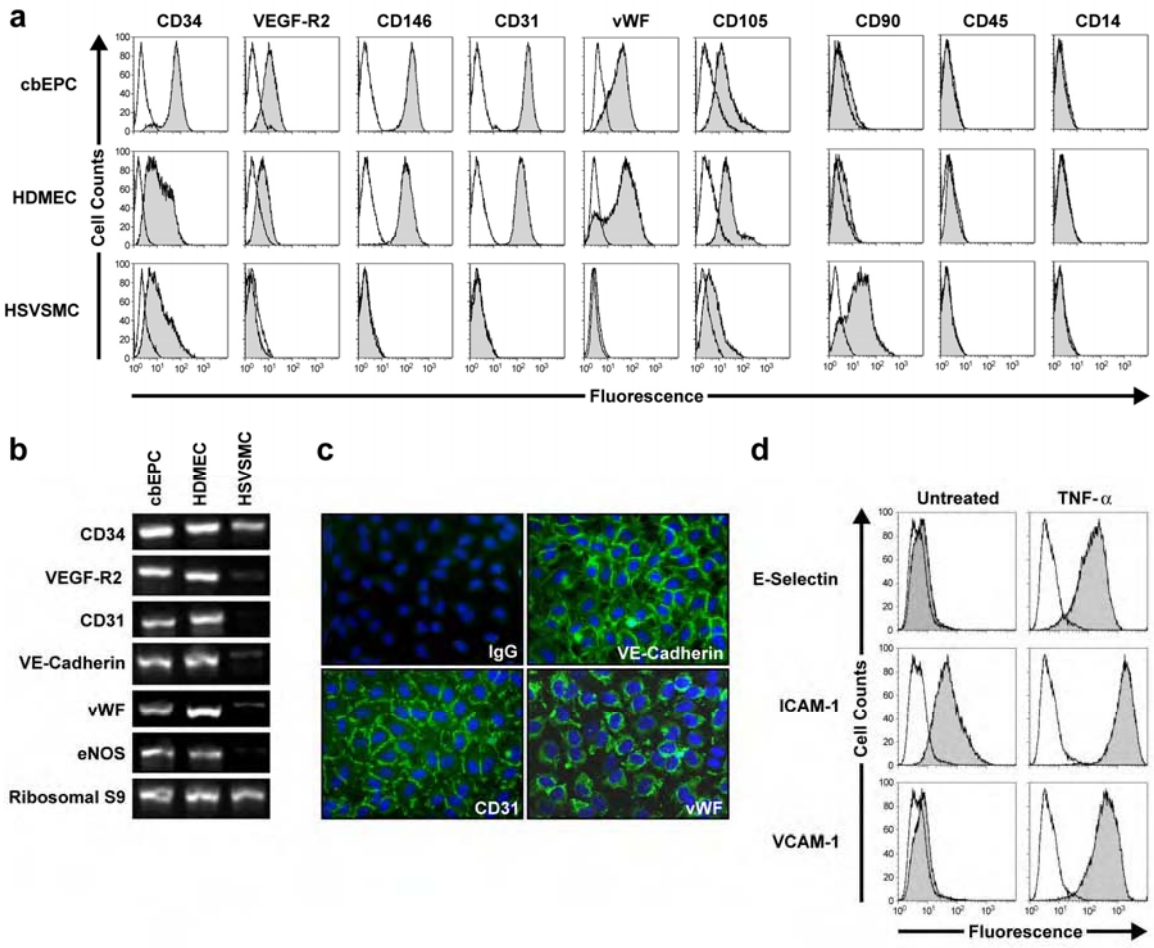
a
CD34
VEGF-R2
CD146
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CD105
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CD14
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HDMEC
HSVSMC
Cell Counts
Fluorescence
b
cbEPC
HDMEC
HSVSMC
CD34
VEGF-R2
CD31
VE-Cadherin
vWF
eNOS
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c
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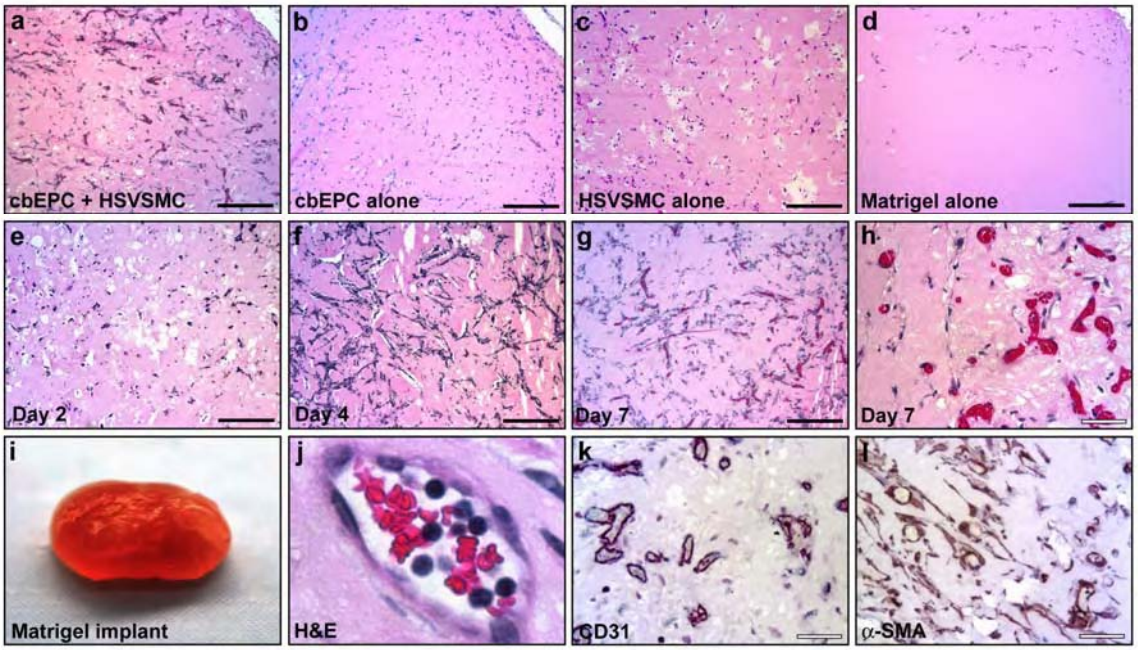
VCAM-1
Cell Counts
Fluorescence

Figure 1


a
cbEPC + HSVSMC
b
cbEPC alone
c
HSVSMC alone
d
Matrigel alone
e
Day 2
f
Day 4
g
Day 7
h
Day 7
i
Matrigel implant
j
H&E
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CD31
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α-SMA

Figure 2

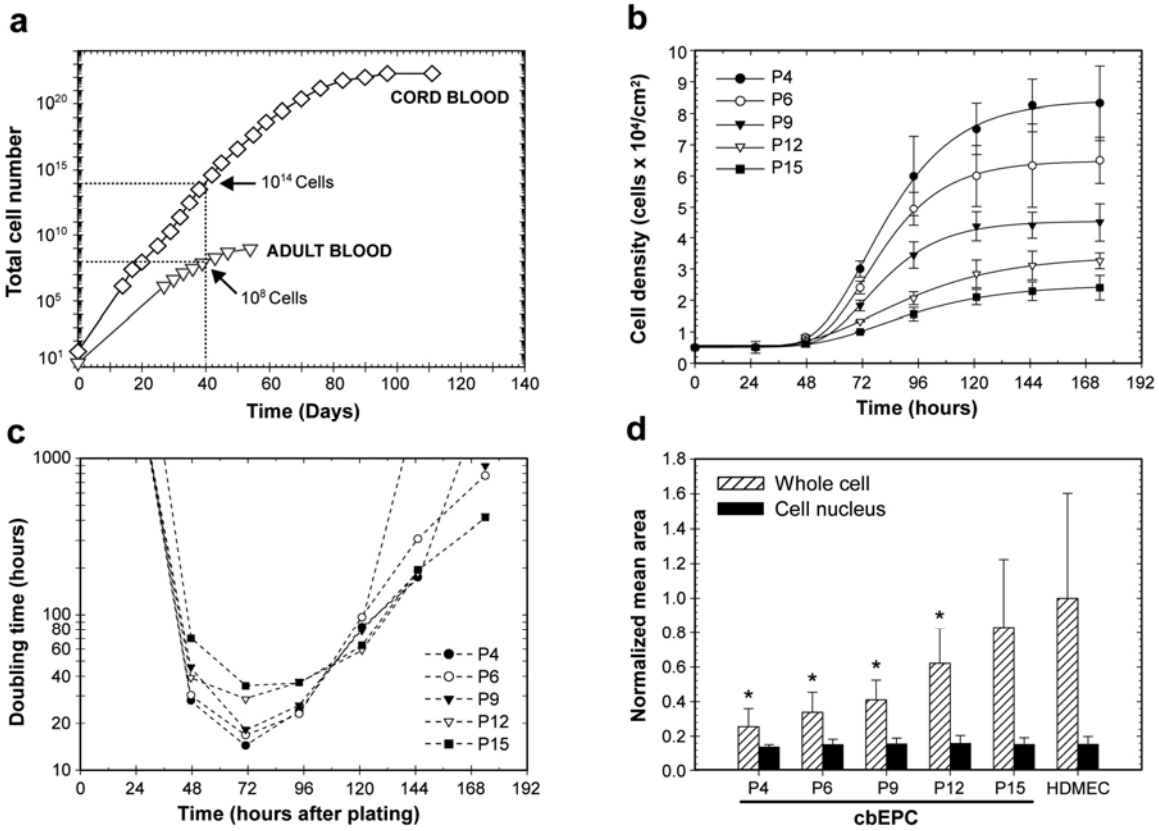
a
Total cell number
Time (Days)
CORD BLOOD
ADULT BLOOD
10^14 Cells
10^8 Cells
b
Cell density (cells x 10^4/cm^2)
Time (hours)
P4
P6
P9
P12
P15
c
Doubling time (hours)
Time (hours after plating)
d
Normalized mean area
Whole cell
Cell nucleus
HDMEC
cbEPC

Figure 3

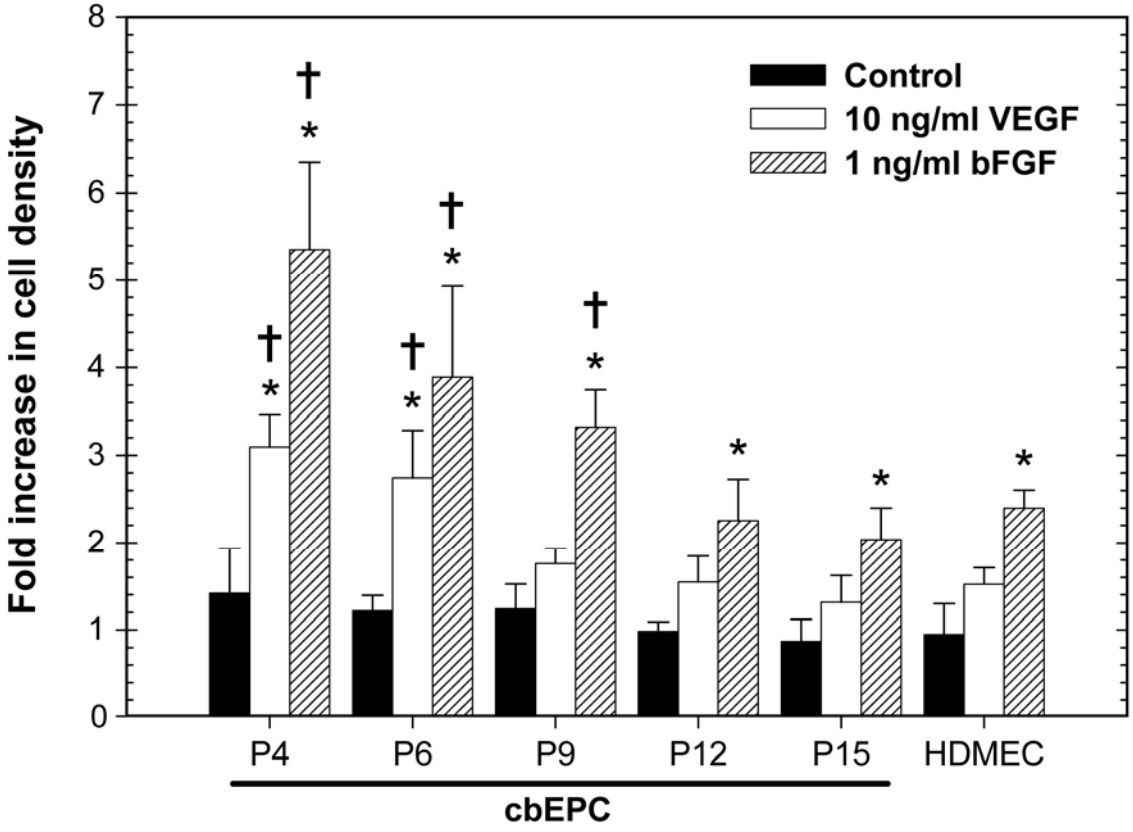
Fold increase in cell density
0
1
2
3
4
5
6
7
8
Control
10 ng/ml VEGF
1 ng/ml bFGF
P4
P6
P9
P12
P15
HDMEC
cbEPC

Figure 4

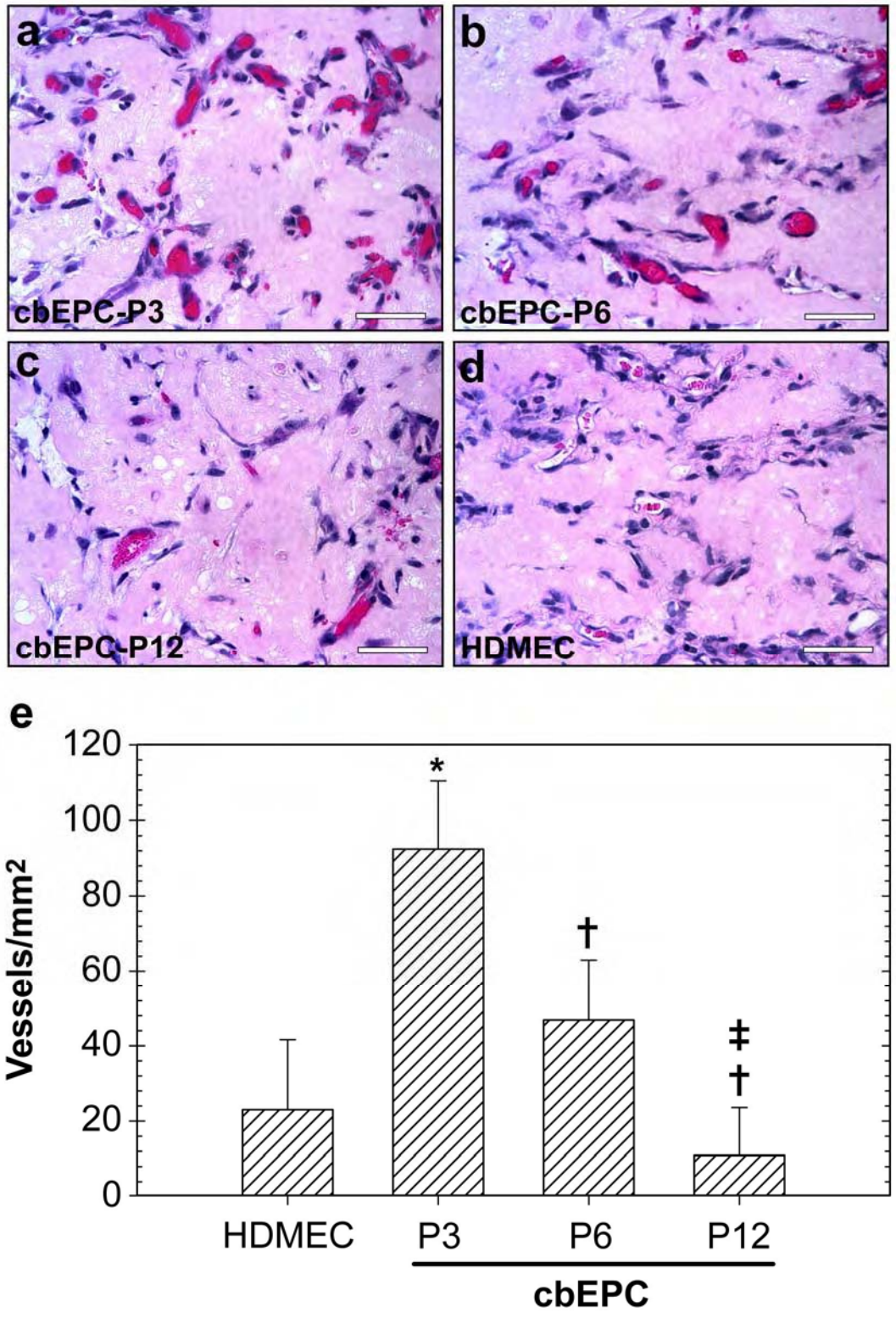
a
cbEPC-P3
b
cbEPC-P6
c
cbEPC-P12
d
HDMEC
e
Vessels/mm²
120
100
80
60
40
20
0
*
†
‡
†
HDMEC
P3
P6
P12
cbEPC

Figure 5

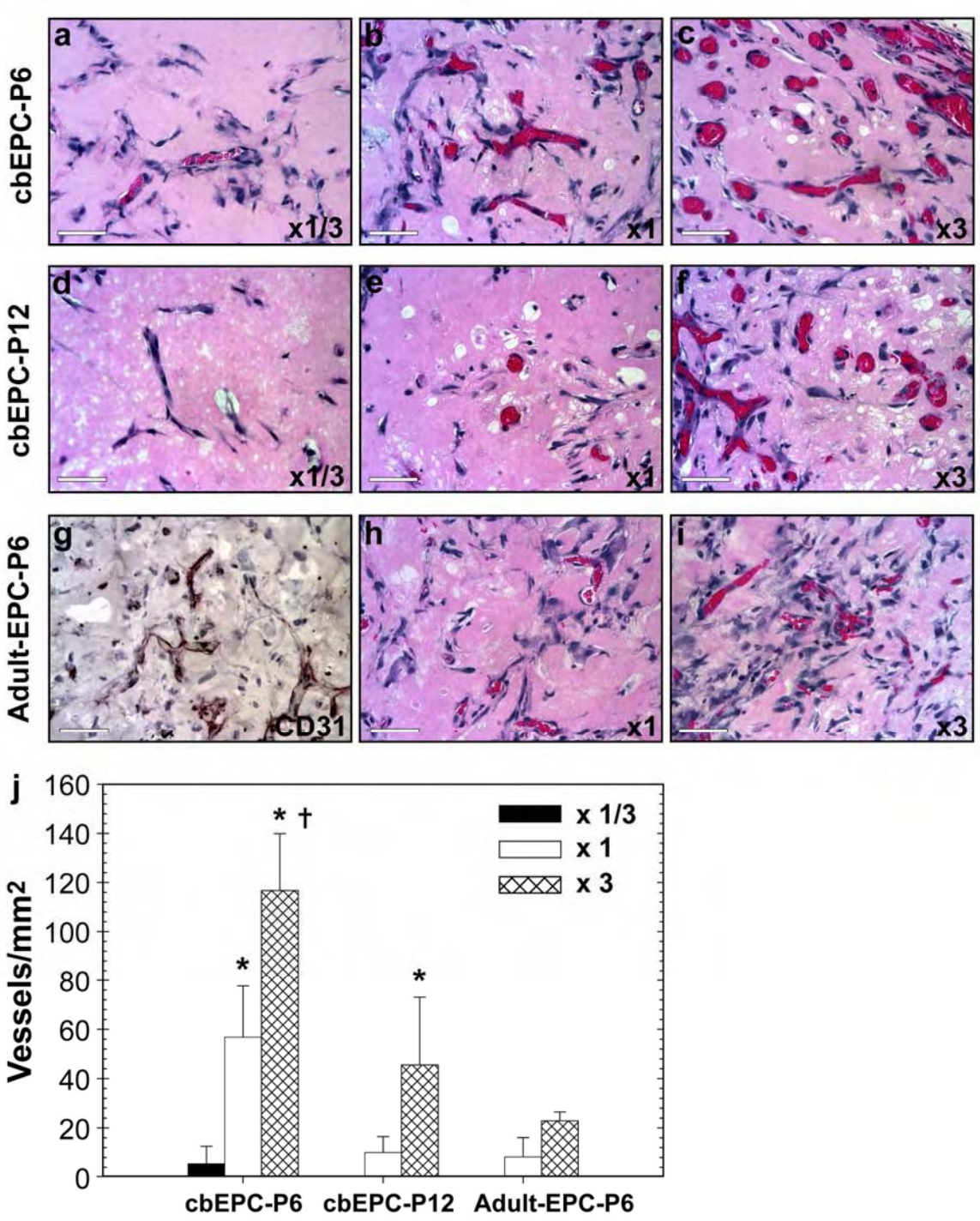
cbEPC-P6
cbEPC-P12
Adult-EPC-P6
a
b
c
d
e
f
g
h
i
x1/3
x1
x3
CD31
j
Vessels/mm²
160
140
120
100
80
60
40
20
0
* †
*
*
x 1/3
x 1
x 3
cbEPC-P6
cbEPC-P12
Adult-EPC-P6

Figure 6